The role of Prokineticin 1 in endometrial function

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Contents i

List of figures xi

List of Tables xv

Declaration xvii

Acknowledgements xviii

Publications and presentations xx

Abstract xxi

Abbreviations xxiii

1. **Chapter 1 - Literature review**

1.1. Introduction 1

1.2. The Endometrium 2

1.3. The menstrual cycle 4

1.3.1. The proliferative phase 4

1.3.2. The secretory phase 7

1.4. The receptive endometrium and implantation 8

1.4.1. Ovarian hormones influence the receptive endometrium 9

1.4.2. Morphological changes to the endometrium 12

1.4.3. Cytokines, growth factors, lipid mediators and protective mechanisms involved in uterine receptivity and implantation 14

1.4.4. Endometrial adhesion molecules expressed in the receptive Endometrium 22

1.4.5. Immune cell colonisation of the secretory phase and early pregnant endometrium 26

1.4.6. Gene array analysis of receptive endometrium and implantation genes implicated by knockout studies 30

1.4.7. The effect of embryonic signals on the endometrium 36

1.5. The prokineticins 38

1.5.1. Prokineticin receptors 39

1.5.2. Tissue localisation and potential functions of the prokineticins 40

1.5.3. Prokineticin mediated signalling 44

1.6. Aims and objectives of this thesis 48

2. **Chapter 2 - General materials and methods**

2.1. Reagents and suppliers 50
2.2. Tissue collection 50
2.2.1. Endometrial tissue 50
2.2.2. Decidua tissue 50
2.2.3. Endometrial cancer tissue 51
2.3. Cell culture 51
2.3.1. PROKR1 receptor transfection and amplification 51
2.3.2. Cell culture conditions 52
2.3.2.1. Passaging of cells 52
2.3.3. Cell and tissue treatments 53
2.3.4. Transient transfection of c-myc ERK, dominant negative isoforms of signalling molecules and COX-2 luciferase Promoter reporter constructs 57
2.3.4.1. Production and characterisation of dominant negatives 57
2.3.4.2.Transient transfection of c-myc ERK and dominant negative isoforms of signalling molecules 60
2.3.4.3.Transient transfection of COX-2 luciferase promoter reporter constructs 61
2.3.4.4.Transient trasfection of COX-2 luciferase promoter reporter constructs and dominant negative isoforms of signalling molecules 62
2.4. Luciferase promoter reporter assay 62
2.5. Polymerase chain reaction 62
2.5.1. RNA extraction 62
2.5.2. Reverse transcriptase polymerase chain reaction 64
2.5.3. Taqman quantitative PCR 65
2.6. Inositol phosphate assay 68
2.7. Western immunoblot analysis 69
2.7.1. Protein extraction and quantification 69
2.7.1.1. Cells 69
2.7.1.2. Tissue 69
2.7.2. Immunoprecipitation 70
2.7.3. Western immunoblot analysis 70
2.7.3.1.Chemifluorescent detection 71
2.7.3.2.Direct fluorescent detection 73

2.8. Immunohistochemistry 75
2.8.1. Tissue fixation, wax embedding and sectioning 75
2.8.2. 3,3'-diaminobenzidine (DAB) immunohistochemistry 75
2.8.3. Double immunofluorescent histochemistry 76
2.9. Gene array 78
2.9.1. Sample preparation 78
2.9.1.1.ABI 79
2.9.1.2.Affymetrix 79
2.9.2. Data analysis 79
2.9.2.1.GO annotations 80
2.9.2.2. EST tissue library 80
2.10. Enzyme linked immunosorbent assays (ELISA) 80
2.10.1. cAMP 80
2.10.2. Prostaglandin 81
2.10.3. Estradiol and progesterone 83
2.10.4. LIF 83
2.11. Statistics 84
2.12. Commonly used solutions 84

3. **Chapter 3 - Expression and localisation of PROK1 and PROKR1 in normal and pathological endometrium**
3.1. Introduction 88
3.2. Materials and methods 89
3.2.1. Tissue collection 89
3.2.1.1.Endometrial tissue 89
3.2.1.2.Decidua tissue 90
3.2.1.3.Endometrial cancer tissue 90
3.2.2. Polymerase chain reaction 90
3.2.2.1.RNA extraction 90
3.2.2.2.Reverse transcriptase PCR 91
3.2.2.3.Taqman quantitative PCR 91
3.2.3. Immunohistochemistry 92
3.2.3.1.DAB immunohistochemistry 92
3.2.3.2.Immunofluorescent histochemistry and confocal microscopy 92
3.2.4. Statistics 93

3.3. Results 93

3.3.1. Expression and localisation of PROK1 in normal cycling endometrium and early pregnancy decidua 93

3.3.1.1.Expression of PROK1 throughout the menstrual cycle 93

3.3.1.2.Expression of PROK1 in first trimester decidua 94

3.3.1.3.Localisation of PROK1 in normal endometrium and first trimester decidua 95

3.3.2. Expression and localisation of PROKR1 in normal cycling endometrium and early pregnancy decidua 98

3.3.2.1.Expression of PROKR1 throughout the menstrual cycle 98

3.3.2.2.Expression of PROKR1 in first trimester decidua 98

3.3.2.3.Localisation of PROKR1 in normal endometrium and first trimester decidua 100

3.3.3. Identification of PROK1/PROKR1 expressing endometrial cells 103

3.3.3.1.Expression of PROK1 and PROKR1 in immune cells within the endometrium 103

3.3.3.2.Expression of PROKR1 in endometrial endothelial cells 105

3.3.4. Expression and localisation of PROK1 in endometrial cancer 106

3.3.4.1.Expression of PROK1 in well, moderately and poorly differentiated endometrial cancer 106

3.3.4.2.Localisation of PROK1 in well, moderately and poorly differentiated endometrial cancer 107

3.3.5. Expression and localisation of PROKR1 in endometrial cancer 107

3.3.5.1.Expression of PROKR1 in well, moderately and poorly differentiated endometrial cancer 107

3.3.5.2.Localisation of PROKR1 in well, moderately and poorly differentiated endometrial cancer 109

3.4. Discussion 111

4. Chapter 4 - Establishment of an Ishikawa endometrial cell line stably expressing PROKR1, and characterisation of PROK1 induced signalling in PROKR1 Ishikawa cells

4.1. Introduction **116**

4.2. Materials and methods **117**

4.2.1. Transfection of PROKR1 cDNA 117

4.2.2. Polymerase chain reaction 118
4.2.3. Taqman quantitative PCR 118
4.2.4. Immunocytochemistry 119
4.2.5. IP assay 119
4.2.6. cAMP assay 119
4.2.7. PROK1 mediated signalling 120
4.2.7.1.PROK1 dose response of ERK 1/2 phosphorylation 120
4.2.7.2.Time course of PROK1 induced ERK 1/2 phosphorylation 120
4.2.7.3.Effect of chemical inhibitors of signalling intermediates on PROK1 induced ERK 1/2 phosphorylation 121
4.2.7.4. Effect of dominant negative isoforms of signalling molecules on PROK1 induced ERK 1/2 phosphorylation 121
4.2.7.5. Time course of PROK1 induced cSrc and EGFR phosphorylation 122
4.2.8. Western immunoblot analysis 123
4.2.9. Statistics 123
4.3. Results **123**
4.3.1. Production of PROKR1 Ishikawa cell line 123
4.3.2. Signalling characterisation of PROKR1 Ishikawa cell line 126
4.3.2.1.PROK1 induced IP mobilisation 126
4.3.2.2.PROK1 mediated cAMP production 127
4.3.2.3.Dose response of PROK1 induced ERK 1/2 phosphorylation 127
4.3.2.4.Time course of ERK 1/2 phosphorylation 128
4.3.3. Mapping the pathway of PROK1-PROKR1 induced signalling to ERK 1/2 129
4.3.3.1.PROKR1 signalling is not Gi coupled 129
4.3.3.2.PROKR1 is Gq coupled and ERK 1/2 phosphorylation is mediated via PLC-β - Ca^{2+} - cSrc - EGFR and MEK 130
4.3.3.3. PROK1 mediated ERK 1/2 phosphorylation is inhibited by dominant negative isoforms of signalling pathway intermediates 132
4.3.3.4. PROK1 induces phosphorylation of cSrc 133
4.3.3.5.PROK1 induces phosphorylation of EGFR 134
4.3.3.6.PROK1 induces phosphorylation of cSrc via Gq and Ca^{2+} 135
4.4. **Discussion** **137**

5. Chapter 5 - PROK1 target genes

5.1. Introduction **141**

5.2. Materials and methods **143**

5.2.1. Cell culture and PROK1 treatments 143

5.2.2. RNA extraction and quantification 144

5.2.3. cRNA preparation and hybridisation to gene chips 145

5.2.3.1.ABI 145

5.2.3.2.Affymetrix 147

5.2.4. Data analysis 148

5.2.5. PCR verification of gene expression 150

5.2.5.1.RNA extraction 150

5.2.5.2.Taqman PCR analysis 151

5.2.6. Statistics 151

5.3. Results **152**

5.3.1. Gene expression altered upon treatment of PROKR1 Ishikawa cells with PROK1 152

5.3.2. Classification of PROK1 regulated genes into gene ontologies 152

5.3.3. Validation of gene expression implicated by gene array 165

5.3.3.1.Validation of gene expression at 8 hours 165

5.3.3.2.Temporal regulation of PROK1 induced gene expression 165

5.4. Discussion **168**

6. Chapter 6 - Prokineticin 1 regulation of COX-2 expression and prostaglandin synthesis in PROKR1 Ishikawa cells

6.1. Introduction **183**

6.2. Materials and methods **184**

6.2.1. Immunofluorescent histochemistry and confocal microscopy 184

6.2.2. Cell culture and treatments 185

6.2.2.1.Time course analysis of PROK1 mediated COX-2 expression 185

6.2.2.2.Mechanism of COX-2 mediated COX-2 expression 185

6.2.3. Polymerase chain reaction 186

6.2.3.1.RNA extraction 186

6.2.3.2.Taqman quantitative PCR 187

6.2.4. Western immunoblot analysis 187

6.2.5. Transient transfection 188
6.2.5.1.Transient transfection of COX-2 luciferase promoter reporter constructs 188
6.2.5.2.Transient transfection of COX-2 luciferase promoter reporter constructs with dominant negative isoforms of signalling molecules 188
6.2.6. COX-2 luciferase assay 189
6.2.7. Prostaglandin ELISA 189
6.2.8. Statistics 190
6.3. Results **190**
6.3.1. Co-expression of PROKR1 and COX-2 in human endometrium and first trimester decidua 190
6.3.2. Temporal regulation of PROK1 induced COX-2 expression 191
6.3.2.1.Temporal regulation of PROK1 induced COX-2 RNA expression in PROKR1 Ishikawa cells 191
6.3.2.2.Temporal regulation of PROK1 induced COX-2 protein expression in PROKR1 Ishikawa cells 192
6.3.3. Mechanism of PROK1 induced COX-2 expression 193
6.3.3.1.PROK1 mediated COX-2 expression is partially transcriptionally regulated 193
6.3.3.2.Mechanism of PROK1 mediated COX-2 transcriptional Activation 195
6.3.3.3.Effect of dominant negative isoforms of signalling molecules on PROK1 mediated COX-2 transcriptional activation 197
6.3.3.4.Mechanism of PROK1 mediated COX-2 RNA expression 198
6.3.3.5.Mechanism of PROK1 mediated COX-2 protein expression 199
6.3.4. De novo synthesis of prostaglandins mediated by COX-2 201
6.3.4.1.PROK1 mediated prostaglandin production is dependent on COX-2 expression 201
6.4. Discussion **203**

7. Chapter 7 - Prokineticin 1 regulation of LIF expression in PROKR1 Ishikawa cells
7.1. Introduction **208**
7.2. Materials and methods **209**

7.2.1. Immunofluorescent histochemistry and confocal microscopy 209
7.2.2. Cell culture and treatments 210
7.2.2.1.Time course analysis of PROK1 mediated LIF expression 210
7.2.2.2.Mechanism of PROK1 mediated LIF expression 210
7.2.3. Taqman quantitative PCR 211
7.2.3.1.RNA extraction 211
7.2.3.2.Taqman quantitative PCR 211
7.2.4. LIF ELISA 212
7.2.5. Statistics 212
7.3. Results **213**
7.3.1. Co-expression of PROKR1 and LIF in human endometrium and first trimester decidua 213
7.3.2. Temporal regulation of PROK1 induced LIF expression in PROKR1 Ishikawa cells 214
7.3.2.1.Temporal regulation of PROK1 induced LIF RNA expression 214
7.3.2.2.Temporal regulation of PROK1 induced LIF protein production 215
7.3.3. PROK1 induced LIF expression is transcriptionally regulated 216
7.3.4. Mechanism of PROK1 induced LIF expression in PROKR1 Ishikawa cells 217
7.4. Discussion **218**

8. Chapter 8 - Physiological relevance of PROK1-PROKR1 signalling in early pregnancy
8.1. Introduction **223**
8.2. Materials and methods **225**
8.2.1. Tissue collection 225
8.2.2. Cell and tissue treatments 225
8.2.2.1.Time course analysis 225
8.2.2.2.Mechanism of PROK1 mediated ERK 1/2 phosphorylation, COX-2 and LIF expression 226
8.2.2.3.Mechanism of hCG induced LIF expression 226
8.2.3. Western immunoblot analysis 227
8.2.4. Polymerase chain reaction 227

8.2.4.1.RNA extraction 227
8.2.4.2.Taqman quantitative PCR 228
8.2.5. LIF ELISA 228
8.2.6. *In vivo* model 229
8.2.7. Statistics 229
8.3. Results **230**
8.3.1. Intracellular signalling activation by PROK1 in first trimester decidua 230
8.3.1.1.Time course of PROK1 mediated ERK 1/2 phosphorylation in first trimester decidua 230
8.3.1.2.Mechanism of PROK1 induced ERK 1/2 phosphorylation in first trimester decidua 231
8.3.2. Gene expression induced by PROK1 in first trimester decidua 232
8.3.2.1.PROK1 induces expression of COX-2 m RNA in first trimester decidua 232
8.3.2.2.PROK1 induces expression of LIF m RNA in first trimester decidua 233
8.3.2.3.PROK1 induces production of LIF protein in first trimester decidua 234
8.3.3. Mechanism of gene expression induced by PROK1 in first trimester decidua 235
8.3.3.1.Mechanism of PROK1 induced COX-2 mRNA expression 235
8.3.3.2. Mechanism of PROK1 induced LIF mRNA expression 236
8.3.4. hCG mediated regulation of PROK1 and LIF expression 238
8.3.4.1.hCG mediated regulation of PROK1 expression in PROKR1 Ishikawa cells, first trimester decidua and *in vivo* model 238
8.3.4.2.hCG mediated regulation of LIF expression in PROKR1 Ishikawa cells and first trimester decidua 240
8.3.4.3.hCG mediated LIF expression may be dependent on PROK1 expression 241
8.4. Discussion **242**

9. Chapter 9 - Final discussion, conclusions and future work
9.1. Final discussion 249
9.1.1. Expression and localisation of PROK1 and PROKR1 in the

Non-pregnant, pregnant and pathological endometrium 249
9.1.2. PROK1 mediated intracellular signalling 251
9.1.3. PROK1 mediated gene expression 252
9.1.4. Relevance to implantation and early pregnancy 255
9.1.4.1.Human endometrial gene expression 255
9.1.4.2.Gene knockout studies 255
9.1.4.3.Endometrial pathologies 256
9.1.4.4.Trophoblast function 257
9.1.4.5.Embryonic signals 258
9.1.5. Potential therapeutic uses for prokineticins or prokineticin inhibitors 258
9.2. Conclusions 261
9.3. Future work 262

List of figures

Figure 1.1. Representation of the female reproductive tract indicating the uterus, the ovary and uterine tube/ oviduct, cervix and vagina. 2

Figure 1.2. Representation of the functionalis and basalis regions of the endometrium 3

Figure 1.3. Representation of the menstrual cycle 5

Figure 1.4. Proposed site of the endometrial stem cells. 6

Figure 1.5. Representation of the indirect effect of Progesterone on epithelial cells. 12

Figure 1.6. Endometrial pinopodes. 13

Figure 1.7. Representation of the stages of apposition and adhesion of the blastocyst. 23

Figure 1.8. Representation of the dynamic variation in endometrial PROK1 expression in concert with Progesterone across the menstrual cycle. 44

Figure 1.9. Schematic indicating Gi coupled receptor signalling 45

Figure 1.10. Schematic indicating Gq coupled receptor signalling 46

Figure 1.11. Schematic indicating Gs coupled receptor signalling 47

Figure 2.1. Dominant negative constructs 60

Figure 2.2. Schematic representing the ECF Western immunoblot detection method. 72

Figure 2.3. Schematic representing fluorescent Western immunoblot detection method. 74

Figure 3.1. Expression of PROK1 across the menstrual cycle. 94

Figure 3.2. Expression of PROK1 in first trimester decidua and comparison with PROK1 expression across the menstrual cycle. 95

Figure 3.3. Localisation of PROK1 in mid-secretory endometrium and first trimester decidua. 97

Figure 3.4. Expression of PROKR1 across the menstrual cycle. 98

Figure 3.5. Expression of PROKR1 in first trimester decidua and comparison with PROK1 expression across the menstrual cycle. 99

Figure 3.6. Localisation of PROKR1 in human endometrium in mid-proliferative, early secretory and mid secretory phases of the menstrual cycle. 101

Figure 3.7. Localisation of PROKR1 in first trimester decidua. 102

Figure 3.8. Expression of PROK1 by uNK cells. 104

Figure 3.9. Localisation of PROK1 to macrophages within decidua. 104

Figure 3.10. Localisation of PROKR1 to macrophages within decidua. 105

Figure 3.11. Localisation of PROKR1 to endothelial cells of the endometrium. 105

Figure 3.12. Expression of PROK1 in endometrial cancer and normal cycling endometrium. 106

Figure 3.13. Localisation of PROK1 in endometrial cancer from well, moderately and poorly differentiated endometrial cancer samples. 108

Figure 3.14. Expression of PROKR1 in endometrial cancer and normal cycling endometrium. 109

Figure 3.15. Localisation of PROKR1 in endometrial cancer from well, moderately and poorly differentiated endometrial cancer samples. 110

Figure 4.1. Semi quantitative and real-time Taqman quantitative PCR expression of PROKR1 in WT and PROKR1 Ishikawa cells. 125

Figure 4.2. Induction of Inositol phosphate mobilisation in WT and PROKR1 Ishikawa cells. 126

Figure 4.3. Induction of cAMP production in response to 40nM PROK1 and forskolin in PROKR1 Ishikawa cells. 127

Figure 4.4. Induction of ERK 1/2 phosphorylation in PROKR1 Ishikawa cells. 128

Figure 4.5. Time course of PROK1 induced ERK 1/2 phosphorylation. 129

Figure 4.6. ERK 1/2 phosphorylation is not inhibited by PTX. 130

Figure 4.7. Chemical inhibitors of signalling intermediates inhibit PROK1 induced ERK 1/2 phosphorylation. 131

Figure 4.8. Inhibition of ERK 1/2 phosphorylation using cDNA constructs encoding dominant negative mutant isoforms of cSrc, EGFR, Ras and MEK. 133

Figure 4.9. PROK1 induced cSrc phosphorylation in PROKR1 Ishikawa cells. 134

Figure 4.10. PROK1 induced EGFR phosphorylation in PROKR1 Ishikawa cells. 135

Figure 4.11. Chemical inhibitors of Gq protein, Ca^{2+} and cSrc but not EGFR

or MEKinhibit cSrc phosphorylation. 136

Figure 5.1. PROK1 mediated gene expression at 8 hours. 166

Figure 5.2. Temporal regulation of PROK1 induced gene expression. 167

Figure 6.1. Co-localization of PROKR1 and COX-2 in endometrium and first trimester decidua. 191

Figure 6.2. Temporal regulation of PROK1 mediated COX-2 RNA expression. 192

Figure 6.3. Temporal regulation of PROK1 induced COX-2 protein expression. 193

Figure 6.4. Inhibition of PROK1 induced transcriptional activation of COX-2. 194

Figure 6.5. PROK1 mediated transcriptional activation of COX-2. 196

Figure 6.6. PROK1 mediated transcriptional activation of COX-2 is dependent on activation of multiple signalling molecules. 198

Figure 6.7. PROK1 mediated COX-2 RNA expression is dependent on activation of multiple signalling molecules. 199

Figure 6.8. PROK1 mediated COX-2 protein expression is dependent on activation of multiple signalling molecules. 200

Figure 6.9. PROK1 induced de novo synthesis of prostaglandins in PROKR1 Ishikawa cells. 202

Figure 7.1. Co-localization of PROKR1 and LIF in mid-secretory endometrium and first trimester decidua tissue. 213

Figure 7.2. Temporal regulation of PROK1 induced LIF RNA expression in PROKR1 and WT Ishikawa cells. 214

Figure 7.3. Temporal regulation of PROK1 induced LIF protein production in PROKR1 Ishikawa cells. 215

Figure 7.4. PROK1 induced LIF expression is transcriptionally regulated. 216

Figure 7.5. Mechanism of PROK1 induced LIF expression. 217

Figure 8.1. PROK1 induced ERK 1/2 phosphorylation in first trimester decidua tissue. 230

Figure 8.2. PROK1 induced ERK 1/2 phosphorylation in first trimester decidua tissue is subject to inhibition by chemical inhibitors of signalling molecules. 232

Figure 8.3. Temporal regulation of PROK1 induced COX-2 expression in first trimester decidua tissue. 233

Figure 8.4. Temporal regulation of PROK1 induced LIF RNA expression in first trimester decidua tissue explants. 234

Figure 8.5. Temporal regulation of PROK1 induced LIF protein production in first trimester decidua tissue explants. 235

Figure 8.6. PROK1 mediated expression of COX-2 in first trimester decidua is dependent on activation of multiple signalling molecules. 236

Figure 8.7. PROK1 mediated expression of LIF in first trimester decidua is dependent on activation of multiple signalling molecules. 237

Figure 8.8. Regulation of hCG mediated PROK1 expression. 239

Figure 8.9. Temporal regulation of hCG mediated LIF expression. 241

Figure 8.10. hCG mediated LIF expression is sensitive to inhibition by inhibitors of protein synthesis. 242

Figure 9.1. Schematic representation of PROK1 mediated signalling and gene activation. 254

List of Tables

Table 1. Endometrial genes up- or down-regulated by gene array analysis. 32

Table 2. Cell density in culture vessels. 53

Table 3. Concentrations of inhibitors. 54

Table 4. Primer sequences for RT-PCR. 65

Table 5. Taqman primer and probe sequences. 68

Table 6. Antibody dilutions used for Western immunoblot detection. 71

Table 7. Tissue processing protocol. 75

Table 8. Antibody concentrations used in DAB immunohistochemistry detection. 76

Table 9. Concentrations of inhibitors of signalling molecules. 121

Table 10. Antibody dilutions used for Western immunoblot analysis. 123

Table 11. Treatment of PROKR1 Ishikawa cells with 40nM PROK1 for 8 hours revealed 277 genes to be regulated with fold changes of greater than 1.5 on each gene array. 153

Table 12. Treatment of PROKR1 Ishikawa cells with 40nM PROK1 revealed 49 genes to be regulated with fold changes of greater than 1.5 on both gene arrays after application of selection criteria of adjusted p values of <0.05. 163

Table 13. Application of gene ontology annotation for biological processes to PROK1 regulated genes revealed 3 significant themes. 164

Table 14. Inhibitor concentrations. 186

Table 15. Inhibitor concentrations. 211

Table 16. Inhibitor concentrations. 226

Declaration

Except where due acknowledgement is made by reference, the studies undertaken in this thesis were the unaided work of the author. No part of this work has been previously accepted for, or is currently being submitted in candidature for another degree

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Abstract

The Endometrium is a dynamic tissue which undergoes cyclical growth in preparation for pregnancy and regression in the absence of pregnancy. In the mid-secretory phase of the menstrual cycle, also known as the window of implantation, the endometrium prepares for the implantation of an embryo by undergoing pre-decidual changes, leukocyte recruitment and increasing the secretory capacity of the epithelial cells. In pregnancy, the embryo secretes human chorionic gonadotropin (hCG), which maintains progesterone production and has direct effects on the endometrium. Prokineticin 1 (PROK1) is elevated during this transient period and may be a mediator of endometrial receptivity.

Prokineticins (PROK1 and PROK2) are pleiotropic proteins with multiple functions including gastrointestinal tract contractility, angiogenesis in the normal and pathological ovary, testis and prostate, noiciceptive sensitisation, transmission of the circadian rhythm of the suprachiasmatic nucleus and immune cell activation. The receptors for the prokineticins (PROKR1 and PROKR2) are two closely related G-protein coupled receptors. Signalling via these receptors is linked to PLC-β activation, inositol phosphate mobilisation, ERK 1/2 and Akt phosphorylation and nitric oxide production.

Investigation of the temporal pattern of expression of PROK1 and PROKR1 in pregnant and non-pregnant endometrium has shown (a) both factors to be expressed in the normal cycling endometrium with an elevation in PROK1 expression during the secretory phase of the cycle and (b) further elevation of both factors in the pregnant decidua when compared with the non-pregnant endometrium. Expression of PROK1 was down-regulated in endometrial cancer tissue compared with secretory phase endometrium, suggesting PROK1-PROKR1 is not involved in the pathogenesis of this disease. PROK1 and PROKR1 localise to the glandular epithelium, stroma and vasculature of the non-pregnant and pregnant endometrium. Additionally, PROK1 expression was localised in macrophages and uterine natural killer cells within the stromal compartment.

In order to investigate signalling and the role of PROK1 in endometrial epithelial cells, an endometrial epithelial cell line (Ishikawa cells) stably expressing PROKR1 was utilised. PROK1-PROKR1 interaction, using this cell line, induced a signalling

cascade involving phosphorylation of cSrc, epidermal growth factor receptor (EGFR) and ERK 1/2. This cascade to ERK 1/2 phosphorylation was dependent on activation of Gq protein, PLC-β and Ca^{2+} as well as phosphorylation of cSrc and EGFR and activation of the small GTPase Ras.

Gene array analysis was subsequently conducted using RNA extracted from PROKR1 Ishikawa cells treated with vehicle or 40nM PROK1 for 8 hours. Gene array analysis was conducted using the Affymetrix GeneChip® Human Genome U133 Plus 2.0 and the ABI 1700 v.2 Applied Biosystems Human Genome Survey microarrays. A total of 277 genes were differentially expressed in response to PROK1 (226 genes were up-regulated and 51 genes down-regulated). A number of these genes have suggested roles in implantation. These include: cyclooxygenase-2 (COX-2), leukaemia inhibitory factor (LIF), Interleukin (IL)-6, IL-11 and Heparin bound-EGF. Two genes, COX-2 and LIF were selected for further investigation in this thesis. In the human endometrium and first trimester decidua, expression of COX-2 and LIF co-localise with PROKR1 to the glandular epithelium and stromal cells. PROK1 induces mRNA expression of both of these factors in a time dependent manner in the PROKR1 Ishikawa cell line and first trimester human decidua. Moreover, prostaglandin production and LIF protein secretion was elevated in response to treatment with PROK1. Using specific inhibitors of cell signalling, the expression of COX-2 and LIF in response to PROK1 was dependent on activation of the Gq-PLC-β-cSrc-EGFR-MEK signalling pathway. Finally, the expression of PROK1 and LIF in the human endometrium can be mediated by embryonic hCG secretion. Treatment of PROKR1 Ishikawa cells and first trimester decidua with 1IU hCG results in sequential increase in PROK1 and LIF expression.

Collectively these data strongly suggest that PROK1-PROKR1 signalling is important (a) in the preparation of the human endometrium for pregnancy by regulating expression of implantation related genes and (b) may perform further functions in early pregnancy under the influence of hCG.

Abbreviations

17βHSD2	17-beta hydroxysteroid dehydrogenase 2
ACE	Adrenal cortex endothelial
ANOVA	Analysis of variance
bp	Base pairs
BSA	Bovine serum albumin
Bv8	Bombina variegata 8
cAMP	Adenosine 3',5'- cuclic monophosphate
CNTF	Ciliary neurotrophic factor
COX	Cyclooxygenase
cPLA2α	Cytosolic phospholipase A2
CSF	Colony stimulating factor
DAB	3,3'- diaminobenzidine
DAF	Decay accelerating factor
Dkk-1	Dickkopf-1
DMEM	Dulbeccos modified eagle medium
DN	Dominant negative
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EGF	Epidermal growth factor
EG-VEGF	Endocrine gland vascular endothelial growth factor
ELISA	Enzyme linked immunosorbent assay
ER	Estrogen receptor
ERK	Extracellular signal regulated kinase
EST	Expressed sequence tag
FCS	Foetal calf serum
FGF	Fibroblast growth factor
GM-CSF	Granulocyte macrophage colony stimulating factor
GnRH	Gonadotropin releasing hormone
GO	Gene ontology
GPCR	G-protein coupled receptor
HB-EGF	Heparin binding EGF-like growth factor
hCG	Human chorionic gonadotropin
HOXA	Homeobox A
HSP	Heat shock protein

HSPG	Heparin sulphate proteoglycan
IgG	Immunoglobulin G
IL	Interleukin
IVF	*In vitro* fertilisation
kDa	Kilodaltons
KGF	Keratinocyte growth factor
KIR	Killer inhibitory receptor
LDL	Low density lipoprotein
LH	Luteinising hormone
LIF	Leukaemia inhibitor factor
MCP	Monocyte chemoattractant protein
MEK	Mitogen associated protein kinase/ extracellular regulated kinase kinase kinase
MHC	Major histocompatibility complex
MIP	Macrophage inflammatory protein
MIT-1	Mamba intestinal toxin
MMP	Matrix metalloproteinase
MOX	Methyloxyamine buffer
nM	Nano molar
NO	Nitric oxide
NOS	Nitric oxide synthase
PBS	Phosphate buffered saline
PCOS	Polycystic ovarian syndrome
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor
PG	Prostaglandin
PLC	Phospholipase C
pM	Pico molar
PP14	Placental protein 14
PPAR	Peroxisome proliferator activated receptor
PR	Progesterone receptor
PROK1	Prokineticin 1
PROK2	Prokineticin 2
PROKR1	Prokineticin receptor 1
PROKR2	Prokineticin receptor 2
PTX	Pertussis toxin

PVDF	Polyvinylidene difluoride
RANTES	Regulated upon activation of normal T-cell expressed and secreted
RGD	Arg – Gly - Asp
RNA	Ribonucleic acid
RTK	Receptor tyrosine kinase
RT-PCR	Reverse transcription polymerase chain reaction
RXR	Retinoid X receptor
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
SPP-1	Secreted phosphoprotein 1
TGF	Transforming growth factor
TNF	Tumour necrosis factor
TNS	Tris neutral saline
TRI	Total RNA isolation
uNK	Uterine natural killer
uPA	Urokinase plasminogen activator
VEGF	Vascular endothelial growth factor
VPRA	Venom protein A
WT	Wild type
IP	Inositol phosphate

Chapter 1 - Literature Review

1.1. Introduction

The endometrium is a dynamic tissue, which undergoes proliferation followed by secretory changes in preparation for embryo implantation each menstrual cycle. Implantation involves interactions between the embryo and the maternal endometrium. This is a highly complex process, which, even after many decades of investigation is still incompletely understood. Implantation of the conceptus within the endometrium inside a finite window of endometrial receptivity is essential for the normal progression of pregnancy to term. Implantation at extra-uterine sites or implantation that occurs outside of the normal window when the uterus becomes refractory to implantation are common causes of early pregnancy loss.

Prokineticin 1 (PROK1) is demonstrated to be elevated during the secretory phase of the menstrual cycle, the putative 'window of implantation' (Battersby, 2004a, Ngan, 2006). PROK1 and Prokineticin receptor 1 (PROKR1) its cognate receptor are expressed in multiple cellular compartments within the endometrium. The focus of this thesis is the examination of the role of PROK1 and PROKR1 in endometrial function and its possible role in pregnancy.

This introduction will give a brief overview of the structure, histology, function and endocrinology of the endometrium throughout the menstrual cycle with a focus on the peri-implantation phase of the menstrual cycle. The peri-implantation phase of the menstrual cycle will be described in terms of the endocrine milieu, morphological changes, the alterations in cytokines, growth factors and lipid mediators, expression of endometrial adhesion molecules, the immune cell population of the endometrium and the changes which take place in the endometrium when it comes under the influence of factors secreted from the blastocyst. This introduction will also cover in detail the known tissue expression pattern of the prokineticins and their receptors, signalling induced by prokineticins and potential functions regulated by the prokineticins in physiological and pathological states.

1.2. The Endometrium

The uterus is a pear shaped organ situated within the pelvic cavity (Figure 1.1). Embryonically the uterus is derived from the fused Mullerian ducts (Koff, 1933). This was first described by Johannes Muller in 1825. The mullerian duct epithelium differentiates to give rise to the luminal and glandular epithelium of the endometrium, while the surrounding urogential ridge mesenchyme differentiates into the endometrial stroma along with the inner and outer myometrial layers (Cunha *et al*, 2002). The uterus is divided into three structural regions, the fundus, the corpus and the isthmus. It also consists of three layers, the underlying perimetrium which is a thin membrane, the myometrium which is a layer of smooth muscle underlying the third layer, the endometrium. The endometrium itself is further divided into specific regions, the stratum basalis and stratum functionalis (Figure 1.2).

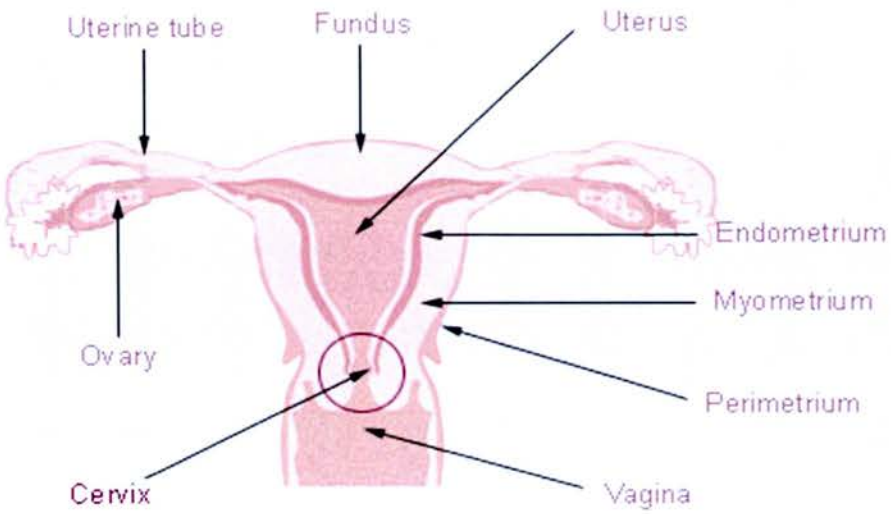


Figure 1.1. Representation of the female reproductive tract indicating the uterus, the ovary and uterine tube/ oviduct, cervix and vagina. Adapted from www.training.seer.cancer .gov

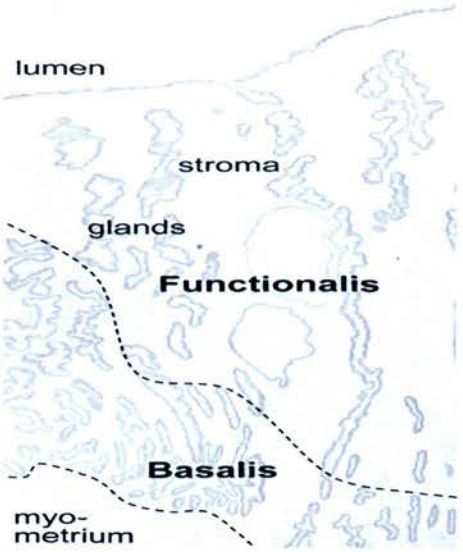


Figure 1.2. Representation of the functionalis and basalis regions of the endometrium, delineated by the dotted lines, and the underlying myometrium layer. Adapted from Gargett, 2007.

The endometrium is an incredibly dynamic tissue, undergoing regeneration and proliferation with formation of new blood vessels, secretory transformation and preparation for pregnancy. This is followed by the degeneration and breakdown of the functionalis at menstruation. Menstruation is only found in a limited selection of mammals, including humans, old world moneys such as chimpanzees, gorillas, orangutans and gibbons, new world monkeys such as capuchin monkeys and a species of bat (Dempsey, 1939, Graham, 1981, Ortiz *et al*, 1995, Ortiz *et al*, 2005). The reason for the development of a sophisticated menstrual cycle within these animals is still unclear. However, a link has been suggested between menstruating mammals and their type of placentation, which is haemochorial, i.e. invasive placenta development. Species that exhibit epitheliochorial placentation i.e. non-invasive placentation, do not menstruate (Enders, 1991, Smith, 2002).

The human menstrual cycle is split into 3 distinct phases, the proliferative or follicular phase, the secretory or luteal phase and the menstrual phase. The function of the endometrium is the provision of a tissue which can support the implantation and development of an embryo. The proliferative and secretory phases therefore represent preparation of the functionalis layer of the endometrium for this function, while menstruation represents the redundancy

of this tissue in the absence of conception after the endometrium becomes refractory to implantation.

1.3. The menstrual cycle

1.3.1. The proliferative phase

Menstruation is initiated in the absence of pregnancy in response to falling estrogen and progesterone concentrations as the corpus luteum degenerates (Figure 1.3). The regeneration of the endometrium is traditionally considered to be under the influence of estrogen (Figure 1.3). However, initial endometrial repair is proposed, based on morphometric data, to be complete within 48 hours after the first two days of menses (i.e. initiation on day 3, Ferenczy, 1976). Maximum proliferation, particularly of the luminal and glandular epithelium occurs on days 3 - 4 of menses, with re-epithelialisation occurring by migrating and spreading of denuded basalis over underlying fibroblasts. Reconstruction of the luminal epithelial barrier is complete by day 5 (Ferenczy, 1979). These initial signs of regeneration occur when estrogen levels are still low. It has therefore been postulated that initial endometrial regeneration takes place from endometrial stem cells resident within the basalis region.

Tissues which undergo self-renewal must be in possession of a lifelong population of stem cells, which are capable of mature cell production and post-injury regeneration (Lemischka, 2001). The endometrium is a proposed site of stem cells within the deep basalis region (Padykula, 1991, Gargett, 2007, Figure 1.4). This was based on the high level of proliferation of the glandular epithelial cells in the basalis (Okulicz *et al*, 1993, Padykula *et al*, 1984, Padykula *et al*, 1989), as a burst of proliferation is characteristic of stem cell to progenitor transition (LeBlond, 1981). Indeed, it was demonstrated in 1944 that, after removal of all visible endometrium, the entire primate endometrium could regenerate (Hartman, 1944) suggesting the presence of stem cells. Support for the non-essential role of estrogen in the initial regeneration of the endometrium is provided by the evidence that on day 3 of menses, when regeneration starts, the glandular and luminal epithelium display little estrogen receptor (ER)α immunoreactivity (Okulicz & Scarrel,

1998). There is also evidence that even in the absence of all estrogen, from ovaries, diet and fat reserves, the endometrium still regenerates (Kaitu'u-Lino *et al*, 2007), however, estrogen does enhance the proliferative effect (Okulicz *et al*, 1997).

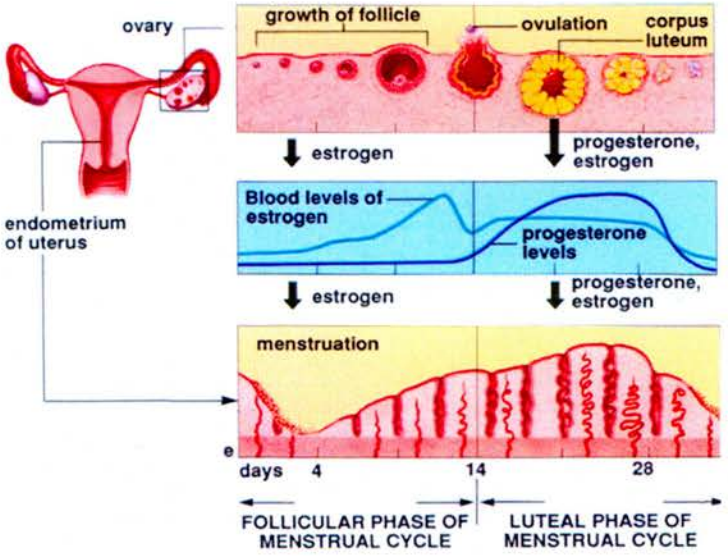


Figure 1.3. Representation of the menstrual cycle indicating follicle growth and rupture, ovarian hormone levels and endometrial development. Shedding of the functionalis layer of the endometrium – menstruation - occurs with the decline in estrogen and progesterone levels in the absence of conception. The endometrium is regenerated, from stem cells present within the basalis region and under the influence of wound healing factors, during the proliferative phase when predominant ovarian steroid hormone is estrogen. During the secretory phase of the menstrual cycle, under the influence of progesterone from the corpus luteum, the endometrium undergoes secretory changes in preparation for pregnancy. During this phase of the cycle an influx of uNK cells is observed, the endometrial blood vessels become spiral arterioles, the glands increase their secretory capacity and become tortuous and the tissue is oedematous. In the absence of pregnancy the endometrium becomes refractory to implantation and the functionalis layer is again shed. Adapted from www.soc.ucsb.edu

It is suggested that the regenerative process is influenced by normal wound healing factors other than estrogen (Johannison *et al*, 1982). Estrogen, however, may stimulate epithelial cell proliferation through a paracrine mediator, possibly through effects on stromal cells. Stromal cell expansion appears to be dependent on the epithelial compartment and estrogen as tissue recombination studies indicate that re-epithelialisation is required for stromal cell

responsiveness (Bigsby, 2002). Expansion is noted on days 5 - 6 of the cycle coincident with an elevation in estrogen and ERα expression (Kaitu'u-lino *et al*, 2007, Ferenczy, 1979). This suggests that the initial epithelial proliferation is derived from estrogen independent proliferation of stem cells, while further proliferative effects may be derived from estrogen mediated paracrine signals from the stromal compartment.

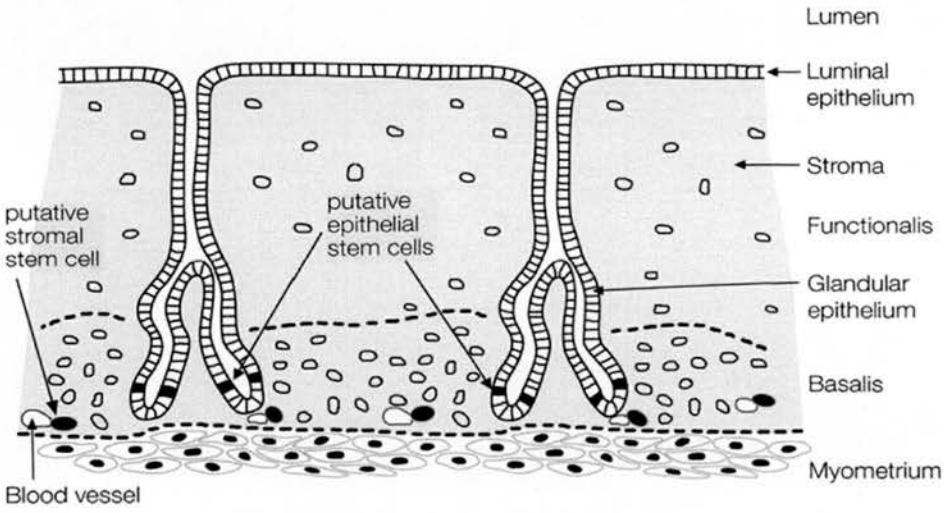


Figure 1.4. Proposed site of the endometrial stem cells. Stem cells which direct regeneration of the functionalis region of the endometrium are proposed to reside within the basalis layer. These proliferate during the early proliferative phase of the cycle to give rise to the new functionalis layer. Adapted from Gargett, 2007.

The proliferative phase 'proper' commences on day 5 of the menstrual cycle. This phase is characterised by the appearance of short narrow glands and cell proliferation, which continues until approximately 3 days post ovulation or after the luteinizing hormone (LH) surge (Tabibzadeh *et al*, 1990, Ferenczy, 1979). Significant endothelial repair and proliferation also takes place during this phase of the cycle (Ludwig & Spornitz, 1991, Ferenczy, 1979). During this repair and proliferation, several growth factors associated with wound healing are induced, including platelet derived growth factor (PDGF), transforming growth factor (TGF)-α, epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), and fibroblast growth factor (FGF) (Werner *et al*, 1992, Brown *et al*, 1992, Greenhalgh, 1996). Several of these growth factors possess chemotactic properties, which attract inflammatory cells to the region and

perpetuate the repair and proliferation response (Witte & Barbul, 1997). Indeed, it is now considered that the action of estrogen on epithelial and stromal cell proliferation is mediated by the induction of growth factor expression in the stromal compartment. Blood vessel growth in the proliferative phase of the cycle is thought to be mediated by EGF (Nelson *et al*, 1991), TGF-α, TGF-β, tumour necrosis factor (TNF)-α (Leibovich *et al*, 1987), FGF-1 and -2 (Moller *et al*, 2001), VEGF (Li XF *et al*, 1994) and PDGF (Moghaddam *et al*, 1995, Zhang *et al*, 1997). By the time of ovulation, at the proliferative - secretory phase transition, when progesterone takes over as the dominant hormone, the endometrium presents a thickness of 3 - 4 mm and displays an undulant surface (Noyes *et al*, 1975).

1.3.2. The secretory phase

During the secretory phase, the endometrium is mainly under the control of progesterone (Figure 1.3). However, estrogen is still present albeit at lower levels than during the proliferative phase. The length of the secretory phase varies from 8 - 17 days (Lenton *et al*, 1984), with the initial secretory changes apparent in the glandular epithelium by cycle day 15 - 16 (Noyes *et al*, 1950). In the early secretory phase, from LH +0 to +7, the level of progesterone rises rapidly and structural changes in the endometrium are rapid. Progesterone inhibits uterine epithelial cell proliferation and induces 17β-hydroxysteroid dehydrogenase (HSD) 2, which metabolises estrogen to its less active derivative estrone, thereby inhibiting its mitogenic effect (Tseng & Gurpide, 1975). The glands change from a relatively undifferentiated state through co-ordinated changes to achieve maximum synthesis and secretory capacity by LH +7 (Dockery & Rogers, 1989, Dockery *et al*, 1988, Li TC *et al*, 1988, Hey *et al*, 1995). These secretory products are thought to be of importance in supporting the trophoblast in the initial stages of invasion (Burton *et al*, 2007).

The secretory phase is characterised by a period of conversion from dominance of the epithelial compartment to dominance of the stromal compartment (Lessey, 2002). There is an influx of large granular lymphocytes, characterised as uterine natural killer cells (uNK), with the presence of macrophages and $CD3^+$ T cells (Spornitz *et al*, 1992, Yeaman *et al*, 1997, Givan *et al*, 1997). These

are thought to have relevance in the preparation of the endometrium for pregnancy. The stromal cells differentiate and undergo pre-decidual changes with elaboration of proteins thought to be critical for implantation success (Lessey, 2002). By the time the endometrium is committed to undergoing decidualization, uNK cells are abundant and are proposed to have effector functions potentially important for the decidualization process (King, 2000a). Oedema becomes apparent on days 20 - 23 of the menstrual cycle, associated with the influx of immune cells, the secretory transformation and decidual changes (Okada *et al*, 2001, Daly *et al*, 1982).

The secretory phase of the menstrual cycle encompasses the period known as the window of implantation. This is a transient phase of approximately 4 days duration. This period represents a state of readiness of the endometrium for implantation, which occurs approximately 7 days after the LH surge (LH +7) or 5.5 days post ovulation (Aplin, 2000). This window is associated with an increase in expression of a number of potential 'markers' of receptivity that are regulated by the endometrial hormonal milieu.

1.4. The receptive endometrium and implantation

The concept of the window of implantation was suggested by Psychoyos in 1973 (Psychoyos, 1973). This proposal of a window of time in which implantation could take place was elaborated in further studies (McLaren, 1973, Finn & Martin, 1974). Use of animal models to investigate an optimal period of time for endometrial implantation aided the definition of this window (McLaren & Mitchie, 1954, Hodgen *et al*, 1983, Psychoyos, 1986). The embryo is capable of adhering to and growing on almost any tissue in the body (Fawcett, 1950, Kirby, 1963), except the uterus when the endometrium, specifically the luminal epithelium, is refractory to implantation (Navot *et al*, 1991). This 'window' of time during which implantation can occur in the uterus, based on pregnancy rates observed after IVF, appears to occur from days 7 - 11 after the LH surge (Bergh & Navot, 1992). However, despite many years of interest, no single marker of uterine receptivity to implantation has been definitively identified in the endometrium.

From a clinical perspective, failure of fertility is a growing problem with approximately 1 in 6 couples affected (Lessey, 2000). Although implantation problems as a cause of infertility are sometimes regarded with scepticism, an emerging trend reveals that inadequate uterine receptivity is responsible for approximately 2/3 of implantation failures. Inadequate uterine receptivity is considered as a major cause of infertility in otherwise healthy women (Achache & Revel, 2006). This may account for the relatively low success rate of IVF at around 25% (de los Santos, 2003).

Endometrial histology dating applying the classical criteria developed by Noyes (Noyes *et al*, 1950) is not accurate, with the day of ovulation based on an increase in basal body temperature. However, this is not true for all women. Application of newer criteria examining the LH surge, presence of pinopodes, or examination of integrin expression (Acosta *et al*, 2000, Lessey, 2000, Shoupe *et al*, 1989) were proposed to date the endometrium more accurately. However, it has been demonstrated that histological endometrial dating does not necessarily correspond with fertility (Coutifaris *et al*, 2004). Molecular and biochemical events are therefore examined in order to gain insight into this period (Sunder & Lenton, 2000). There is much debate over markers which define the endometrial window of implantation. However, this phase may be described by a number of parameters including, enhanced cytokine and growth factor production, production of lipid mediators, expression of adhesion molecules on the luminal epithelium and elaboration of extracellular matrix molecules, a change in morphology, infiltration of bone marrow derived cells and stromal differentiation. By animal knockout studies and gene array analysis of peri-implantation endometrium a number of modulators of endometrial receptivity have been proposed. In the event of pregnancy the endometrium changes further as it comes under the influence of proteins secreted from the blastocyst. The factors involved in receptivity and implantation will now be discussed in more detail.

1.4.1. Ovarian hormones influence the receptive endometrium

Regulation of endometrial differentiation, in order to become receptive and support development and implantation of an embryo, is orchestrated by the sequential actions of estrogen and progesterone (Huet-Hudson *et al*, 1989).

The roles of the steroid hormones are complex and still incompletely understood (Punyadeera *et al*, 2003). Hormonal activity depends on expression of their respective receptors and the levels of each steroid hormone, with the appropriate cyclical pattern of steroid receptor expression being crucial for achieving receptivity (Lessey *et al*, 2003, Ma *et al*, 2003). In the proliferative phase of the menstrual cycle, the ER isoforms, ERα and ERβ, are expressed in the glands; stroma and luminal epithelium with ERβ also expressed in the endothelial cells (Critchley *et al*, 2001, Matsuzaki *et al*, 1999, Witek *et al*, 2001). Progesterone receptor (PR)α is dominant over PRβ expression in the glandular epithelium during the proliferative phase of the menstrual cycle (Mote *et al*, 1999, Diedrich *et al*, 2007). Under the influence of estrogen, ER and PR are elevated in the epithelium (Liu *et al*, 2003, Mote *et al*, 1999, Diedrich *et al*, 2007).

During the secretory phase of the cycle, regulated by progesterone, expression of both ER and PRα are down-regulated in the epithelial compartment with elevation of PRβ in the stroma (Lessey *et al*, 1996, Mote *et al*, 1999). This down-regulation in PR expression in the epithelium appears to be a conserved function in mammals and may allow for a switch from ovarian steroid regulation of the epithelium to paracrine regulation by the progesterone sensitive stroma (Lessey, 2000, Figure 1.5). Down-regulation of epithelial PR appears to characterise the opening of the window of implantation, and persistence of PR expression in women with luteal phase defect is associated with the loss of other markers of receptivity in the epithelium (Lessey *et al*, 1996), suggesting the direct effect of progesterone on the epithelium may serve to inhibit certain markers of receptivity. Progesterone appears to regulate expression other epithelial markers of receptivity such as pinopod formation. However the formation of pinopodes (discussed in Section 1.4.2) is suggested to be dependent on a down-regulation of PR expression (Stavreus Evers *et al*, 2001).

Progesterone appears to be required for functions associated with receptivity such as decidualization (Mulac-Jericevic *et al*, 2000). Synthetic progestagens elevate expression of inducible nitric oxide synthase (iNOS) and production of

nitric oxide (NO), which is thought to contribute to myometrial quiescence during pregnancy (Cameron *et al*, 1998). The influx of leukocytes in the secretory phase of the menstrual cycle is suggestive of steroid control, and progesterone regulates expression of immune related genes suggesting an effect on the uterine immune environment. However, as the uterine immune cells do not possess steroid hormone receptors (Poropatrich *et al*, 1987, Loke & King, 1995), this effect on leukocyte recruitment is probably indirect (King, 1996 *et al*, Makrigiannakis *et al*, 2006).

Studies in humans and non-human primates using anti-progestins further highlight the proposed role of progesterone in preparing the endometrium for implantation. Leukaemia inhibitory factor (LIF), which has been demonstrated as being essential for implantation (Stewart *et al*, 1992), is down-regulated by anti-progestins (Cameron *et al*, 1997, Puri *et al*, 2000, Gemzell Danielsson *et al*, 2003, Sengupta *et al*, 2003, Danielsson *et al*, 1997). This is highly interesting when considering that the regulation of LIF by progesterone is still debated (Kholkute *et al*, 2000, Arici *et al*, 1995, Hambartsoumian *et al*, 1998a, Ace *et al*, 1995, Keltz *et al*, 1996). Treatment of endometrial explants with the progesterone receptor antagonist, mifepristone, also down-regulates signalling molecules activated by cytokines including LIF (Catalano *et al*, 2003). This renders the endometrium non-receptive (Critchley *et al*, 1999) suggesting cytokines and down-stream signalling activation are required for endometrial receptivity and implantation.

Mifepristone also affects other proposed mediators of endometrial receptivity including cyclooxygenase 2 (COX-2) and Glycodelin (Gemzell Danielsson, 2003 *et al*, Marions & Danielsson, 1999, Sengupta *et al*, 2003). Bonnet monkeys treated with progesterone antagonist, onapristone, also display a down-regulation in epithelial expression of integrins $\alpha v\beta 3$ and $\alpha 1\beta 1$, which are thought to be important for mediating attachment of the blastocyst to the epithelium (Puri *et al*, 2000). A study employing a gene array approach examined endometrial expression of genes in women who had an intrauterine contraceptive device fitted and found 147 genes to be dysregulated during the window of implantation compared with controls. 52 of these genes were

characterised implantation genes including LIF and glycodelin (PP14, placental protein 14), which have previously been shown to be down-regulated by anti-progestins (Horcajadis *et al*, 2006, Cameron *et al*, 1997, Gemzell Danielsson *et al*, 2003, Sengupta *et al*, 2003, Danielsson *et al*, 1997). These data highlight the importance of progesterone, whether through direct or indirect action, in preparing the endometrium for implantation.

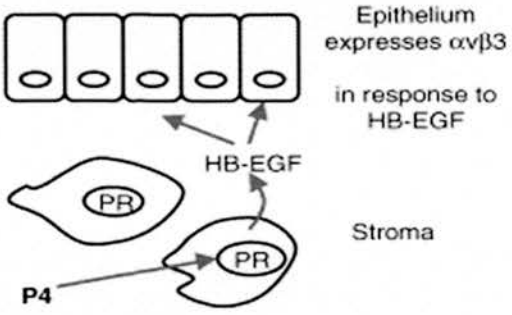


Figure 1.5. Representation of the indirect effect of Progesterone on epithelial cells. Progesterone responsive stromal cells release signals to mediate paracrine effects on the epithelium (P4 – progesterone, PR – progesterone receptor, HB-EGF – heparin binding EGF). Adapted from Sharkey & Smith, 2003, Best Prac & Res Clin Obstet Gynecol.

1.4.2. Morphological changes to the endometrium

One of the major morphological changes in the endometrium thought to characterise the period of endometrial receptivity is the development of pinopodes on the luminal epithelium. These were originally described in mice (Nilsson, 1958) and rats (Warren & Enders, 1964, Fainstat & Chapman, 1965). The term 'pinopod' from the Greek which means 'drinking foot' was applied to these structures representing their pinocytotic function. In humans several differences exist, they appear to arise from the entire apical cell surface and may not be pinocytic, thus the term uterodome was proposed (Guillomot *et al*, 1986, Murphy, 2000, Adams *et al*, 2001), however the term pinopode is still widely used.

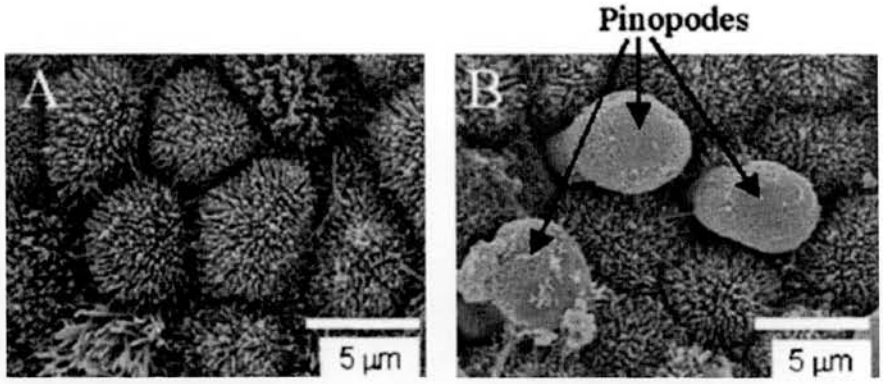


Figure 1.6. Endometrial pinopodes. A indicates normal endometrial microvilli extending from the surface of individual cells. B indicates pinopodes with smooth surfaces protruding above the endometrial microvilli. Adapted from Quinn *et al*, 2007.

Pinopodes are bleb-like protrusions on the apical surface of the endometrium (Usadi *et al*, 2003). These project above the level of the microvilli into the uterine lumen (Figure 1.6). Pinopodes are present for around 2 days of the menstrual cycle in the period between 6 - 10 days after progesterone exposure (Nikas *et al*, 1995) corresponding to the proposed window of implantation (Stavreus-Evers *et al*, 2001, Aghajanova *et al*, 2003). Pinopodes are therefore thought to be progesterone dependent; Homeobox A (HOXA)-10 is also thought to have a role in pinopode development (Lopata *et al*, 2002, Bagot *et al*, 2001). A delay in the expression of pinopodes potentially represents a poor pregnancy outcome (Edwards, 1995). Development of pinopodes on the apical surface is thought to be associated with a loss of polarity, which allows the epithelia of the trophectoderm and endometrium to adhere (Thie *et al*, 1996, Denker, 1990). Pinopodes appear to be preferential sites of embryo - endometrial interaction (Achache & Revel, 2006) and it is suggested that, by extracting fluid from the uterine lumen, the pinopodes facilitate the movement of the blastocyst towards the uterine epithelium by 'drawing' the blastocyst in. However it is unclear if this occurs in humans as they are not thought to be pinocytic (Adams *et al*, 2002). Attachment of embryos in culture to human epithelial monolayers does however appears to occur by interaction with pinopodes (Bentin-Ley *et al*, 1999). It is therefore proposed that pinopodes are a useful morphological marker of uterine receptivity (Nikas & Psychoyos, 1997). However a recent study has suggested that as pinopodes are dependent

on progesterone they do not define the window of implantation and may be detected into pregnancy (Quinn *et al*, 2007). The appearance of pinopodes is also associated with the expression of other putative markers of receptivity including LIF and LIF-receptor (Aghajanova *et al*, 2003, Kabir-Salmani *et al*, 2005, Fouladi-Nashta *et al*, 2005), loss of PR and appearance of integrin αvβ3 (Lessey *et al*, 1992). Pinopodes also appear to express growth factors and cytokines associated with receptivity, which are discussed in Section 1.4.3.

1.4.3. Cytokines, growth factors, lipid mediators and protective mechanisms involved in uterine receptivity and implantation.

The endometrial milieu of hormones, cytokines, growth factors, lipid mediators and protective factors present during the window of implantation is an area which has provided a vast amount of information on how the endometrium prepares itself for the embryo. Clearly in humans, due to ethical considerations, little information can be derived about the actual implantation site. However, much information has been provided by histological studies, examination of uterine lavage and gene array analysis of receptive endometrium. With the advent of technology that allowed genes to be knocked out *in vivo*, the knowledge of genes involved in the mechanism of implantation has advanced greatly. Although the implantation mechanisms in rodents and humans are not identical, with estrogen being an absolute requirement for implantation in rodents, and differences in placentation, parallels may be drawn with genes expressed in the human endometrium. Although not completely equivalent, knockout animals allow genes to be studied, *in vivo*, in way that could never be achieved in humans.

A number of cytokines, especially cytokines of the interleukin (IL)-6 family, are proposed to be important in receptivity and implantation. Broadly, cytokines are proteins and peptides that are used as signalling compounds. The roles of LIF, IL-6, IL-11 and IL-1 in the endometrium will be discussed.

The proposed role of LIF in endometrial receptivity arose from initial investigations in the mouse. In the mouse model it was demonstrated that on day 4 of pregnancy, coincident with the surge of nidatory estrogen, LIF

expression was induced in the endometrial glands occurring prior to implantation, which takes place on midnight of day 4. It was also shown in this study that LIF expression did not depend on the presence of a blastocyst as the elevation in LIF expression also occurred in pseudopregnant mice (Bhatt *et al*, 1991). The role for LIF in implantation was confirmed by use of the LIF null mouse model. Blastocysts were produced and developed in the LIF null mouse, however they did not display any signs of implanting and only developed weak attachments with the luminal epithelium exemplified by the demonstration that these blastocysts could be flushed from the uterine lumen (Stewart *et al*, 1992). In this study maternal control of LIF expression was confirmed as the defective component.

Extensive work examining the human endometrium has now characterised the cycle-dependent expression of LIF with expression in the mid-secretory phase of the menstrual cycle (Charnock-Jones *et al*, 1994, Kojima *et al*, 1994, Arici *et al*, 1995, Culinan *et al*, 1996, Licht *et al*, 1998). LIF is expressed on pinopodes (Kabir-Salmani *et al*, 2005), which, as discussed in section 1.4.2, are potentially the first points of contact between the endometrium and the embryo. The blastocyst expresses the two LIF receptor heterodimers - LIF-R and gp130 (Charnock-Jones *et al*, 1994, Sharkey *et al*, 1995, Van Eijk *et al*, 1996). LIF potentially enters into a reciprocal relationship with the developing embryo as human chorionic gonadotropin (hCG), one of the first embryonic products, induces endometrial expression of LIF (Licht *et al*, 2001, Perrier d'Hauterive *et al*, 2004, Sherwin *et al*, 2007). LIF has also been demonstrated to induce expression of hCG and syncytial transformation (Sawai *et al*, 1995a, 1995b). In human reproduction it has been demonstrated that women who display unexplained infertility with multiple failures of implantation display lower levels of LIF in uterine flushings (Laird *et al*, 1997, Hambartsoumian, 1998b). It has recently been suggested that levels of LIF may be used as a predictor of reproductive outcome, with a higher level of LIF in uterine flushings associated with reproductive success (Mikolajczyk *et al*, 2007).

LIF is structurally related to IL-6 and is part of the same family of related cytokines along with Oncostatin M, Ciliary neurotrophic factor (CNTF) and IL-

11, which signal through heterodimerisation of their own receptor with the common receptor gp130. IL-6 is expressed within the endometrial luminal and glandular epithelium during the mid - secretory phase of the menstrual cycle (Tabibzadeh *et al*, 1995, Vandermolen & Gu, 1996). IL-6 receptors are expressed on the blastocyst (Nishino *et al*, 1990, van Eijk *et al*, 1996), however a soluble form of the IL-6 receptor (sIL-6R) is suggested to be present in the endometrium of fertile women (Rie *et al*, 1995). The formation of a complex between sIL-6R and IL-6 could induce heterodimerisation with gp130 on the blastocyst to induce signalling (Matsuzaki *et al*, 1995). Similar to LIF, endometrial IL-6 may have a reciprocal relationship with the developing embryo as hCG can induce endometrial expression of IL-6 (Sherwin *et al*, 2007), and IL-6 may stimulate production of hCG from the blastocyst (Matsuzaki *et al*, 1995). It is also suggested that IL-6 plays a direct role in implantation as the IL-6 null mouse displays compromised implantation and development (Robertson *et al*, 2000). IL-6 is also proposed to play a role in trophoblast growth and placentation (Nishino *et al*, 1990). Further evidence for the role of IL-6 in receptivity and implantation is provided by the observation that IL-6 expression is lower in women who suffer recurrent miscarriage (Jasper *et al*, 2007).

IL-11, as indicated, is another member of this family and has also been proposed to have a role in implantation. In the human IL-11 and its receptor IL-11R have been identified in the epithelial and stromal cells of the pregnant and non-pregnant endometrium, and in the trophoblast of the primate (Chen *et al* 2002, 2002, Cork *et al*, 2002, Dimitriadis *et al*, 2002, 2003, Karpovich *et al*, 2003, van Rango *et al*, 2004). The expression of the associated common receptor, gp130, required for heterodimerisation has been described above. The IL-11R null mouse has demonstrated a requirement for IL-11 signalling in decidualization as, even though blastocysts implant into the uterus of the mother a defective decidualization response is displayed resulting in dissolution of the pregnancy before term (Robb *et al*, 1998, Bilinski *et al*, 1998).

The IL-1 system is also thought to be of importance in implantation. In mouse models, administration of IL-1 receptor antagonist (IL-1ra) prevented

implantation (Simon *et al*, 1994). This is postulated to be due, in part, to the action of IL-1 on stimulation of integrin expression in the endometrium (Simon *et al*, 1997). Indeed it has been demonstrated that IL-1ra is downregulated in the human endometrium during the window of implantation compared to the proliferative phase (Simon *et al*, 1995). This suggests a mechanism whereby the antagonism of IL-1 is removed thereby allowing its pre-implantation actions (Boucher *et al*, 2001).

A number of growth factors also have proposed roles in endometrial function at the time of implantation. Growth factors are naturally occurring proteins, which are capable of stimulating cellular proliferation and differentiation. The growth factors discussed herein, are colony stimulating factor (CSF)-1 and the EGF family of growth factors. PROK1 is a growth factor with a potential role in implantation; however, as PROK1 is the focus of this thesis it will be discussed in more detail in section 1.5.

CSF-1 was demonstrated to stimulate the proliferation and differentiation of monocytes, and was subsequently found to be expressed by fibroblasts, monocytes, macrophages and endothelial cells (Zolti *et al*, 1991). CSF is abundantly produced by glandular epithelial cells at the proposed time of uterine receptivity (Kauma *et al*, 1991) potentially contributing to the influx of immune cells at this time. The receptor for CSF-1, c-fms is expressed on the embryo and early placental tissues (Pampfer *et al*, 1992, Jokhi *et al*, 1993) indicating potential for interaction during implantation. Mice null for CSF-1, are infertile due to an implantation defect (Pollard *et al*, 1991), reinforcing the hypothesis that endometrial CSF-1 is necessary for implantation. Similar to the factors discussed above, the embryo itself may modulate the endometrial environment in preparation for its own implantation as hCG induces endometrial expression of CSF-1 (Licht *et al*, 1998). Other chemokines such as regulated upon activation normal T-cell expressed and secreted (RANTES), macrophage inflammatory protein (MIP)-1α and -1β, and monocyte chemoattractant protein (MCP)-1 also act to recruit macrophages and granulocytes and can initiate their activation (Simon *et al*, 1998). It is suggested that a surge in these chemokines during the window of implantation

may also contribute to the initiation of implantation (Wood *et al*, 1997) indicating that the endometrial stroma is richly populated with immune cells, their contribution is discussed more detail in Section 1.4.5.

Another 'family' of factors implicated in the regulation of implantation is the EGF family of molecules, these include EGF itself, TGF-α, amphiregulin, cripto, heparin binding-EGF (HB-EGF) and betacellulin. EGF is present in the endometrium throughout the menstrual cycle with a shift from stromal expression in the proliferative phase of the cycle where it may mimic the action of estrogen (Nelson *et al*, 1991), to luminal and glandular expression in the secretory phase of the cycle (Hofmann *et al*, 1991). EGF is postulated to play a role in trophoblast invasion as it downregulates connexin 40 (a transmembrane protein) and increases invasion (Wright *et al*, 2006). Downregulation of connexin 40 is associated with a switch from a proliferative to an invasive phenotype of extravillous trophoblasts (EVT, Wright *et al*, 2006). Potential for paracrine signalling exists as the blastocyst expresses the EGF receptor (Smotrich *et al*, 1996).

Amphiregulin is potentially involved in implantation, however, mouse models provide much of this data and little is known about its role in the human. Amphiregulin is elevated in the diestrous phase of the cycle (Lee DS *et al*, 2005) and is expressed on day 4, the day of implantation in mice, in the uterine epithelium (Carson *et al*, 2000) localised specifically around the site of blastocyst attachment (Das *et al*, 1995). However, by day 5 of pregnancy amphiregulin is absent (Wang & Armant, 2002). Expression of amphiregulin is thought to be under the control of progesterone (Das *et al*, 1995). However, it was noted in the LIF null mouse model that the expected pattern of amphiregulin expression was absent even though progesterone levels were normal (Song *et al*, 2000), indicating amphiregulin may be under the control of local factors rather than steroid hormones. Amphiregulin expression has been determined in the rhesus monkey and peaks on day 16 - 20 of the menstrual cycle (Yue *et al*, 2000); its role in the human however is currently unclear. It has been suggested to play a role in human implantation (Giudice *et al*, 1999) and

has been localised to the endometrial leukocytes and stromal cells (Ejskjaer *et al*, 2005).

HB-EGF has been heavily implicated in the initiation and continuation of pregnancy. HB-EGF is elevated in the mouse luminal epithelium 6 hours prior to attachment (Das *et al*, 1994) and could mediate trophoblast attachment as the spatiotemporal expression suggests an interaction (Das *et al*, 1994). In the human HB-EGF localises to the luminal epithelium, specifically to the pinopodes (Stavreus-Evers *et al*, 2002) and is expressed specifically during the window of implantation (Leach *et al*, 1999, Yoo *et al*, 1997). Interactions between the endometrium and the embryo may be possible via HB-EGF binding to heparin sulphate proteoglycans (HSPG, Raab & Klagsbrun, 1997), which are present in the surface of the blastocyst after acquisition of attachment competence (Carson *et al*, 1993, Smith *et al*, 1997). Interactions may also occur between endometrial HB-EGF and the EGF receptor ErbB4 on the trophoblast (Chobotova *et al*, 2002a). Indeed, it has been demonstrated that cells expressing the transmembrane form of HB-EGF can adhere to active (ie. have acquired attachment competence) but not dormant blastocysts (Raab *et al*, 1996). As indicated for amphiregulin, expression of HB-EGF is absent in the luminal epithelium of the LIF null mouse at the expected time of the attachment reaction (Song *et al*, 2000). The blastocysts in LIF null mice display very weak interactions with the luminal epithelium and they can be easily flushed and retrieved from the uterine cavity (Stewart *et al*, 1992). Therefore it may be presumed that LIF induced elevation of HB-EGF may be necessary for the formation of adequate adhesion between the blastocyst and the epithelium. That LIF expression has been found to be lower in women with unexplained fertility with implantation defects (Laird *et al*, 1997, Hambartsoumian, 1998b) may point towards a role for this cytokine - growth factor cascade in human reproduction. The role of signalling induced by the EGF family growth factors in implantation is further reinforced by the demonstration that EGF receptor null mice on various genetic backgrounds display placental abnormalities (Sharkey, 1998, Threadgill *et al*, 1995).

The lipid mediators produced by the action of COX-2, the prostaglandins are proposed to be essential to female reproduction, in processes from ovulation to parturition (Plunkett *et al*, 1975, Friel *et al*, 2005). The role of COX-2 and the prostaglandins in the endometrium during receptivity and implantation is discussed below.

Although definitive expression of COX-2 has not been demonstrated, by gene array analysis, to be up-regulated during the window of implantation there is a great deal of evidence pointing towards its role in implantation. As indicated, COX-2 does not appear to be up-regulated during the window of implantation, however it is expressed at the luminal epithelium in both humans (Jones *et al*, 1997, Marions & Danielsson, 1999, Stavreus-Evers *et al*, 2005) and rodents (Lim *et al*, 1997) at the expected time of implantation. Indeed, in mice this expression of COX-2 can be more specifically pin-pointed to the luminal epithelium and stroma underlying the blastocyst at time of the attachment reaction on day 4 of pregnancy (Chakraborty *et al*, 1996). Examination of the COX-2 null mouse model has revealed defects and delays in implantation, decidualization and parturition (Lim *et al*, 1997, Cheng & Stewart, 2003). These defects were found to be due to a lack of downstream prostaglandin production as administration of exogenous prostaglandin analogues could partially rescue the defects (Lim *et al*, 1997).

The cytoplasmic phospholipase A2 null mouse model has provided further evidence of the requirement for prostaglandins in implantation. Cytoplasmic phospholipase A2α (cPLA2α), an enzyme involved in the release of arachidonic acid, is essential for prostaglandin synthesis from membrane phospholipids (Clark *et al*, 1995). The involvement of COX-2 and downstream prostaglandins in implantation is reinforced by the observation that mice which are null for cPLA2α display a delay in implantation, a defect in embryo spacing defective placentation and smaller litters (Song *et al*, 2002, Ye X *et al*, 2005, Shah & Catt, 2005), similar to the phenotype of the COX-2 null mouse model. The impact for a potential delay in implantation due to a deficiency in COX-2 derived prostaglandins is illustrated by the observation in humans that

a delay in implantation, beyond the normal window of implantation results in an increased risk of early pregnancy loss (Wilcox, 1999).

A role for prostaglandins in the human has been suggested by the observation that COX-2 is necessary for decidualization of human stromal cells (Frank *et al*, 1994). An increase in vascular permeability in the endometrium is consistently observed in association with implantation, and it has been demonstrated that COX-2 derived prostaglandins are mediators of this permeability (Chakraborty *et al*, 1996). Regulation of COX-2 expression may or may not be under the control of progesterone. Studies have reported that COX-2 staining increases after progesterone withdrawal (Hapangama *et al*, 2002) and, conversely, that anti-progestins decrease COX-2 staining (Marions & Danielsson, 1999). Regulation of COX-2 expression is also thought to be under the control of the implanting blastocyst, and hCG has been demonstrated to play a role in regulating endometrial expression of COX-2 (Han *et al*, 1996, Han *et al*, 1999, Zhou *et al*, 1999). It appears, however, that there may also be a maternal component involved in COX-2 regulation as COX-2 expression is deficient in LIF null mice, which are infertile due to a maternal defect (Stewart *et al*, 1992, Song *et al*, 2000, Fouladi - Nashta *et al*, 2005). As indicated above, LIF defects have been observed in women with unexplained infertility and aberrant COX-2 expression may contribute to this defect.

Protective and stress induced molecules are expressed in the endometrium at the expected time of implantation and are thought to play a role in preventing toxic damage to the endometrium.

Heat shock proteins (HSP24, HSP27, HSP70), α-crystalline B-chain (another member of the heat shock protein family), and metallothioneins are present in the endometrium during the window of implantation and are thought to confer protection (Tabibzadeh & Broome, 1999, Jaattela *et al*, 1993, Jaquier - Sarlin *et al*, 1994). They can potentially act as molecular chaperones during implantation for other molecules required at this time (Sunder & Lenton, 2000, Mehlen *et al*, 1995a, Sies, 1993). All these factors are elevated during the

window of implantation (Gruidl *et al*, 1997, Ciocca *et al*, 1983, Borthwick *et al*, 2003, Carson *et al*, 2002, Kao *et al*, 2002, Mirkin *et al*, 2005, Riesewijk *et al*, 2003, Tabibzadeh *et al*, 1996, Talbi *et al*, 2006). HSP expression is also present in the decidua during the first trimester of pregnancy (Neuer *et al*, 1999). The heat shock protein family member α-crystalline B-chain is also elevated in the baboon endometrium upon treatment with hCG (Sherwin *et al*, 2007).

1.4.4. Endometrial adhesion molecules expressed in the receptive endometrium

The luminal epithelium is considered to be a barrier to implantation as the blastocyst can only adhere and implant during a narrowly defined window of implantation when the endometrium is receptive (Cowell, 1969). The endometrium however is not the only component, the blastocyst must also become appropriately developed, hatch from its surrounding zona pellucida to reveal the trophectoderm and acquire adhesion competence (Paria *et al*, 1993). Adhesion may therefore be considered as a dialogue between the embryo and the maternal endometrium, which cannot be initiated until both have reached an appropriate stage of development (Figure 1.7). Initially the blastocyst and the luminal epithelium come into close contact upon blastocyst hatching, a stage defined as apposition. This is followed by the formation of firm contacts with the endometrium known as adhesion (Kimber & Spanswick, 2002). The adhesive molecules expressed by the endometrium are key to this process and will now be discussed further.

One of the initial contacts between the mother and the embryo is thought to occur via the pinopodes, as discussed in Section 1.4.2. Briefly, pinopodes are bleb-like protrusions on the apical surface of the endometrium (Usadi *et al*, 2003) which are thought to draw in the blastocyst by extracting fluid from the uterine lumen and interact with the blastocys, however this 'drawing in' may not occur in humans (Adams *et al*, 2002). The two polarised epithelial surfaces are usually non-adhesive, however, there is a marked reduction in the apical-basal polarity of the luminal epithelium at the time of the attachment reaction (Denker, 1990, Thie *et al*, 1996). This reduction in polarity facilitates adhesion of these surfaces.

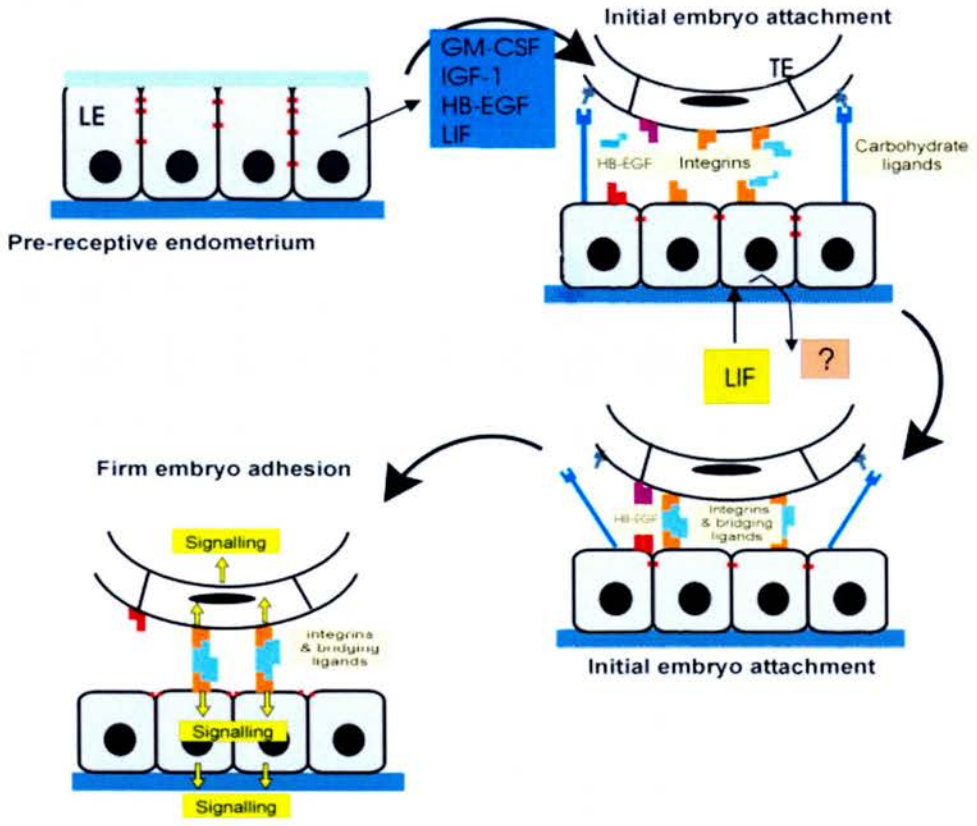


Figure 1.7. Representation of the stages of apposition and adhesion of the blastocyst. TE – trophoblast, LE – luminal epithelium. Molecules potentially involved in mediating the adhesion are shown. In the initial phases of adhesion, loose connections are formed between the endometrium and blastocyst via endometrial HB-EGF, CD44 and Galectins. Firm adhesions are formed between endometrial and blastocyst integrins, proposed to occur by a sandwich model with ECM bridging ligands. Adapted from Aplin & Kimber, 2004, Reprod Biol Endocrinol.

The initial maternal-foetal dialogue is thought to involve interactions including maternal HB-EGF with embryonic EGF receptor (EGFR) and heparin sulphate proteoglycan (HSPG), galectins, CD44 and selectins. As discussed above, HB-EGF is expressed at the luminal epithelium during the window of implantation and has been specifically localised in the human endometrium to the pinopodes (Stavreus-Evers *et al*, 2002), which, as indicated are implicated in initial attachment. HB-EGF can interact with HSPG, which forms around the blastocyst after acquisition of adhesion competence (Carson *et al*, 1993, Smith *et al*, 1997) and EGFR present on the blastocyst surface (Raab *et al*, 1996, Rapolee *et al*, 1998, Dardik *et al*, 1992). Indeed, it is proposed that HSPG actually facilitates the binding of HB-EGF to the EGFR ErbB4 (Higashiyama *et*

al, 1993, Paria *et al*, 1999) thereby promoting adhesion. LIF may also play a role in this interaction as LIF is also present on the pinopodes (Kabir-Salmani *et al*, 2005), and may control expression of HB-EGF (Song *et al*, 2000). Blastocysts form only very loose connections with the luminal epithelium in LIF null mice (Chen JR *et al*, 2000). Expression of LIF at the luminal epithelium may therefore provide another means of adherence. Pinopodes, therefore, appear to play a dual role in adhesion, initially by potentially 'drawing in' the blastocyst and also by promoting adhesive interactions via adhesive molecules.

CD44 is elevated in the endometrium during the window of implantation (Behzad *et al*, 1994, Horne *et al*, 2002) on the luminal epithelium and is also expressed in the pre-implantation embryo (Campbell *et al*, 1995) but not the invading trophoblast. CD44 may mediate adhesive interactions in different ways by binding to two different molecules; hyaluronic acid, which is present in tissues undergoing rapid growth like the blastocyst, or osteopontin. It is suggested that osteopontin, which is expressed in the endometrial glands during the secretory phase (Apparaoe *et al*, 2001), can bind CD44 (Weber *et al*, 1996), and may act as a bridging ligand for αv integrins present on the endometrial and blastocyst epithelium.

Galectins are a group of soluble lectins, which are thought to mediate trophoblast attachment. Galectin-1 is thought to modulate interactions between integrins and ECM components during skeletal muscle differentiation (Gu *et al*, 1994) and could potentially regulate such interactions during implantation (Vicovac *et al*, 1998, Maquoi *et al*, 1997). Selectins are well established as mediators of leukocyte transendothelial trafficking (Alon & Feigelson, 2002), where they allow tethering and rolling of leukocytes before firm adhesion and transmigration. An analogy can be drawn between this process and blastocyst adhesion (Genbacev *et al*, 2003, Dominguez *et al*, 2005). L-Selectin expression is found over the whole surface of the blastocyst (Genbacev *et al*, 2003) while the selectin ligands are present on the luminal epithelium (Lai *et al*, 2005). It is proposed that these may aid the blastocyst in determining the best place to stop and adhere (Achache & Revel, 2006).

Once the blastocyst has formed initial contacts with the endometrium, firm attachment must then be established in order for the blastocyst and the maternal endometrium to become intimately associated and initiate invasion. This is proposed to occur mainly by integrin ECM binding. Expression of the molecules involved in these two systems must be synchronised if an attachment competent blastocyst is to implant into an appropriately receptive uterus.

The integrins are among the best characterised markers of uterine receptivity (Lessey, 1998). Integrins are glycoprotein receptors for a variety of components of the extracellular matrix (ECM) and are composed of α and β subunits which heterodimerize to form functional integrin receptors for ECM ligands. The integrins behave as modulators of cellular function through attachment and signal transduction (Giancotti & Ruoslahti, 1999). The integrins have been proposed as factors which could potentially be used to define the window of implantation. Indeed, expression of α1β1, α4β1, αvβ3 and αvβ5 have been suggested to 'bookend' the window with endometrial expression noted from cycle days 20 - 24 (Tabibzadeh *et al*, 1992, Lessey *et al*, 1992, Lessey *et al*, 1994, Aplin *et al*, 1996). In fertile women the expression of α4 has been demonstrated as being elevated from the time of ovulation and disappears around cycle day 24, while β3 is elevated from around cycle day 20 (Acosta *et al*, 2000, Gonzalez *et al* , 1999, Lessey *et al*, 1992, Illera *et al*, 2003). A failure to express a normal array of endometrial integrins has been demonstrated in many infertile patients with normal histology or those suffering from endometriosis, hydrosalpinges and polycystic ovarian syndrome (Lessey *et al*, 1995, Lessey, 2002, Meyer *et al*, 1997, Ota *et al*, 1997, Tei *et al*, 2003). The significance of this however is unknown as it has been reported that no significant differences are observed in αvβ3 expression and pinopod formation in infertile women (Creus *et al*, 2002), and the mouse null mutant for αvβ3 and humans deficient in β3 are fertile (Hodivala-Dilke *et al*, 1999). These mice however, do show placental defects. This may imply a certain degree of redundancy in this system and it is now accepted that use of integrins alone is insufficient to date the endometrium or completely explain infertility (Lessey, 2000). It is recognised, however, that endometrial integrins identify and bind

ECM components with Arg-Gly-Asp (RGD) sequences, which have been implicated in trophoblast ECM adhesion and outgrowth (Armant *et al*, 1986, Yelian *et al*, 1995), suggesting a sandwich model of integrin adhesion.

Clearly, if any of these mechanisms are to take place, the elaboration of the appropriate integrin ligands is required. ECM components including fibronectin, collagen IV, laminin and osteopontin are elevated during the window of implantation in the endometrium (Wewer *et al*, 1986, Rider *et al*, 1992, Aplin *et al*, 1988, Wewer *et al*, 1985, Garcia *et al*, 2004). These have been localised to the implantation site in the baboon endometrium (Fazleabas *et al*, 1997, BOR). Endometrial ECM components have been shown to support trophoblast growth *in vitro* (Armant *et al*, 1986, Carson *et al*, 1988).

Thus during the peri-implantation phase of the menstrual cycle the endometrium expresses a range of adhesion molecules, which may be utilised by the blastocyst in the event of pregnancy, in order to facilitate initial endometrium - embryo interactions. Therefore, in order for the endometrium to become receptive to a hatched blastocyst the appropriate complement of endometrial adhesion molecules must be expressed.

1.4.5. Immune cell colonisation of the secretory phase and early pregnancy endometrium

The immune cell population within the endometrium is dynamic with dramatic differences in cell populations between the proliferative and secretory phases. Further changes are observed during pregnancy. During the proliferative phase of the menstrual cycle few leukocytes are observed. However, by the secretory phase of the cycle leukocytes account for 20 - 30% of the total stromal cell number (Hunt, 2002) with around 55% of these being uNK cells (Dockery, 2002) and macrophages accounting for around 20% (Trundley & Moffett, 2004). This proportion increases to represent 40% of the decidua in the first trimester of pregnancy (Trundley & Moffett, 2004). These immune cells modulate the endometrial environment in the initiation and maintenance of pregnancy, however, in the absence of conception they play roles in the involutional changes through which the endometrium must pass in

order to undergo menstruation (Critchley *et al*, 2001). In the event of pregnancy the leukocyte population undergoes rapid changes, which diminish the leukocytes of the adaptive immune system and promote the leukocytes of the innate immune system.

Macrophages are present within the endometrium throughout the menstrual cycle. Their numbers are observed to increase in the early secretory phase of the menstrual cycle, potentially in preparation for implantation, with further increases observed in the late-secretory phase of the menstrual cycle, when they are potentially involved in the initiation of menstruation (Salamonsen *et al*, 2002). The range of cytokines secreted by macrophages depends on their activation status (Gordon *et al*, 1995). The steroid hormone milieu is proposed to determine the macrophage secretory phenotype (Hunt & Robertson, 1996). However it is more likely that this is an indirect control mechanism via local stromal cell paracrine mediators as sex steroid receptors are not detectable on any of the immune cells within the endometrium (Poropatrich *et al*, 1987, Loke & King, 1995, King *et al*, 1996). Macrophages are thought to play a major role in tolerance of the invading trophoblast. They are proposed to phagocytose trophoblast debris, act as antigen presenting cells or affect implantation by producing or responding to cytokines (Rai & Regan, 2002) as their numbers are elevated in the decidua basalis at the implantation site whereas only a few are found within the decidua parietalis. Their role as antigen presenting cells is proposed to involve presenting trophoblast antigens to T-cells whose cytokine production would then act to control the immune response, which may be mounted against the trophoblast (Trundley & Moffett, 2004). Macrophage cytokines themselves are central to activation and suppression of lymphocyte proliferation and function and are regulators of local immunological events. However, it is the local endometrial milieu that controls their activation and therefore their function. Macrophages may also act in a synergistic manner to amplify each other's activation.

Great interest has been directed towards the uNK and their role in the endometrium by reproductive biologists. The uNK cells present within the endometrium display a different phenotype to the majority of circulating

blood natural killer cells with these being CD56-CD16+ (Koopman *et al*, 2003) while natural killer cells within the endometrium are CD56+CD16-. This led to them being assigned the name of uterine natural killer cells (Starkey *et al*, 1988, King *et al*, 1991, Nishikawa *et al*, 1991, Geiselhart *et al*, 1995). A small subset of peripheral blood NK cells exhibits this phenotype (Nagler *et al*, 1989). The interest in uNK cells arose when it was observed that they appeared immediately after ovulation (Salamonsen *et al*, 2002) and comprised over half the endometrial leukocyte population at this time. Further increases in cell populations occur specifically within the first trimester to comprise 70% of the leukocyte population, which persists only until week 20 of gestation (Bulmer *et al*, 2005).

At the time of decidualization, uNK cells are abundant and are suggested to play a role in the decidualization process (King, 2000a). UNK cells express LIF, GM-CSF and CSF-1 (Saito *et al*, 1993 *et al*, Jokhi, 1994a, 1994b, 1994c). Another factor thought to be essential for pregnancy, IL-11, is required for decidual-specific uNK cell maturation (Ain *et al*, 2004). These data may suggest a contribution of the uNK cells towards the preparation of the endometrium for implantation (Salamonsen *et al*, 2002). Human uNK cells produce NO, polypeptide growth factors and angiogenic inducers such as VEGF (Leonard *et al*, 2006, Li XF *et al*, 2001, Tayade *et al*, 2006, Hanna *et al*, 2006, Hunt *et al*, 1997). These factors can potentially promote maturation of maternal arterioles, placental growth and development and guide or direct maternal angiogenesis in a hypoxic environment (Carmeliet, 2003, 2005, Croy *et al*, 1997) towards extravillous trophoblast and anchoring trophoblast, as uNK cells are highly motile cells with receptors for trophoblast antigens (Hanna *et al*, 2003, Hiby *et al*, 2004). Hypoxia is thought to contribute to the regulation of VEGF expression in uNK cells and their interaction with the endothelium (Leonard *et al*, 2006). VEGF mediates adhesion of uNK cells to the vascular basement membrane and endothelial cells, possibly via fibronectin binding (Burrows *et al*, 1995) only to developing rather than established vessels suggesting non-innervated segments of vessels require uNK cell support (Chen WS *et al*, 2002, Leonard *et al*, 2006).

The function of the uNK cells however is still unclear. That they interact with the trophoblast within the decidua basalis and are found clustered thickly around maternal arterioles is accepted (Trundley & Moffett, 2004). It is thought that they may regulate in some way the implantation process and modulate placental development in order to ensure that neither under- or over-invasion takes place in order to avoid rejection or parasitism (Loke & King, 1995), as the trophoblast expresses receptors for the cytokines and growth factors expressed by the uNK cells (Jokhi *et al*, 1993, 1994a, King, 1995, Hanna *et al*, 2006). The uNK cells may therefore be important determinants of reproductive outcome.

From their name it may be considered that the uNK cells may attack the partially foreign invading trophoblast, which is only part 'self' as it expresses both maternal and paternal proteins. The trophoblast is extra-embryonic tissue which expresses endogenous retroviral products, oncofetal proteins and imprinted genes. From the maternal immune system perspective the fetal tissues do not fit into any category of non-self, allogeneic, foreign or infected (Moffet *et al*, 2004, Trundley & Moffett, 2004). It is therefore considered that, depending on the maternal uNK cell receptor repertoire and the trophoblast antigen profile, a form of allorecognition may occur (King *et al*, 1998). The trophoblast express major histocompatibility complex (MHC) genes, HLA class I, which are known to be uNK cell ligands. Trophoblast expresses HLA-C, HLA-E and HLA-G. HLA-C is polymorphic and expression of the paternal allele is suggested to be required for trophoblast recognition (Loke & King, 1995, Hiby *et al*, 2001, Trundley & Moffett, 2004, King *et al*, 2000b). HLA-G in complex with HLA-E has high affinity for activating and inhibitory receptors on the uNK cells (Vales-Gomez *et al*, 1999). Killer inhibitory receptors (KIR) on the uNK cells, which are specific for HLA-C, and CD94 on natural killer cells, for which HLA-E is a ligand, influence the development of the human placenta (Hiby *et al*, 2004, Dietl *et al*, 2006). HLA-G is thought to protect against uNK cell lysis by modulating the uNK cell cytokine profile thereby exerting an immunosuppressive effect (Trundley & Moffet, 2004, Dietl *et al*, 2006).

It is suggested that the overall result of interaction with the trophoblast is an inhibition of the cytotoxic phenotype with a shift to non-killing uNK cells. Indeed uNK cells have low cytotoxicity to phenotypic NK cell targets (Manaseki *et al*, 1989). Some activities of the uNK cells however appear to be essential to adequate placental development. Depletion of uNK cells due to deficiencies in influencing cytokines results in malformation of maternal vessels in insufficient trophoblast invasion leading to placental failure (Guimond *et al*, 1997). The function of the uNK cells may therefore be to neither advance nor retard the development of the placenta and maternal vessels but to modulate development depending on expression of HLA class I molecules, uNK cell receptors and cytokines present in the endometrial milieu. Only uNK cells and macrophages have the opportunity to encounter the trophoblast and interact with it thus potentially modulating its behaviour and influence the overall reproductive outcome. As indicated above a number of chemokines are present in the endometrium, which may initiate the initial recruitment of these immune cells into the endometrium, to which the endometrial vascular permeability may contribute. Once present they may produce cytokines to perpetuate this recruitment into pregnancy where they perform functions essential to the development of pregnancy (Ashkar *et al*, 2003).

1.4.6. Gene arrays on receptive endometrium and implantation genes implicated by knockout studies

By gene knockout studies and gene array analysis examining expression of genes during the window of implantation (LH+ 2-4 VS LH+ 6-10 overall range) a number of factors not thus far discussed have been implicated in the regulation of uterine receptivity and implantation.

1.4.6.1.Gene array studies of mid-secretory phase endometrium

In a comparison of six independent studies examining gene expression differentially regulated during the window of implantation, compared with the early secretory phase of the menstrual cycle, it was found that a consensus of at least 3 arrays demonstrated significant elevation of 19 genes with significant down-regulation of 6 genes. The individual gene

arrays demonstrated differential regulation of a greater number of genes but due to differences in study design (e.g. Comparison of different days after the LH surge) and differences in methodology (e.g. Pooled vs. non-pooled samples, different gene array platforms) only a small number of genes displayed consensus among the arrays. These data are summarised in Table 1. These genes will now be discussed in more detail with reference to their roles in receptivity and implantation.

Annexins are a superfamily of proteins that bind to calcium and phospholipids and therefore may alter phospholipase activity and regulate prostaglandin production, specifically $PGF_{2\alpha}$ production (Myatt *et al*, 1992, Schatz *et al*, 1986). This may be important for myometrial quiescence as $PGF_{2\alpha}$ induces contractions of the myometrium (Friel *et al*, 2005), which may be detrimental to maintenance of pregnancy.

Apolipoprotein D (ApoD) is required for lipid metabolism, which is controlled throughout endometrial development and regulated specifically during the window of implantation (Horcajadas *et al*, 2007) while anti-progestins alter the endometrial lipoprotein profile (Benagiano *et al*, 2004, Teran *et al*, 1987). ApoD may act as a transporter molecule for progesterone and arachidonic acid for cellular function and prostaglandin synthesis (Rassart *et al*, 2000). Prostaglandins, as discussed in Section 1.4.3 are thought to be important for implantation and decidualization.

Expression of complement protein 1r (C1r) may be linked with expression of another gene on the list, Ser (Cys) Clade G (C1) inhibitor member 1 (SERPING1). SERPING1 is an estrogen responsive gene in mice (Reese *et al*, 2001), which encodes a plasma protein that has been shown to regulate the first component of C1r by inhibition of the proteolytic activity of its subcomponents (Davis *et al*, 1986) and is therefore involved in the immune response.

Decay accelerating factor or CD55 (DAF) is reduced in women with luteal phase defect and antiphospholipid syndrome, a condition associated with

implantation defects and early miscarriage (Kaul *et al*, 1995, Backos *et al*, 1999, Wilson *et al*, 1999). DAF is suggested to mediate protection against lysis by complement.

Table 1. Endometrial genes up- or down-regulated by gene array analysis. Six individual gene arrays were conducted; these data represent the consensus genes significantly changed on at least 3 arrays. Adapted and amended from Wang & Dey, 2006, Nature Rev Genetics.

Gene	Molecule encoded/ putative function	LH+ (8-10) vs. LH+ (4-6) Kao, 2002	LH+ (7-9) vs. LH+ (2-4) Carson, 2002	LH+ 7 vs. LH+ 2 Riesewijk, 2003	LH+ (6-8) vs. LH+ (3-5) Borthwick, 2003	LH+ 8 vs. LH+ 3 Mirkin, 2005	MSE vs. ESE Talbi, 2006
Up-regulated							
ANXA4	Annexin4	+		+		+	+
AOPD	Apolipoprotein D	+	+	+	+		+
BNIP2	BCL-2/ cell death protein		+	+	+		
CLDN4	Claudin-4	+	+	+			+
C1R	Complement protein 1r	+			+	+	
DAF	Decay accelerating factor for complement	+		+	+	+	+
DF	Complement factor D	+		+	+		
DKK1	Dickkopf-1	+	+	+	+		+
GADD45A	Growth arrest and DNA damage inducible protein	+		+	+	+	
GBP3	Guanylate binding protein 2		+	+	+		
ID4	Inhibitor DNA binding 4	+		+		+	+
IL15	Interleukin 15	+	+	+		+	+
MAP3K5	Mitogen activated protein kinase kinase kinase 5	+		+	+	+	
MT1	Metallothionein 1 family proteins	+	+	+	+	+	+
MAOA	Monoamine oxidase A	+		+	+	+	+
PAEP	Progestagen associated endometrial protein	+		+	+		+
RARRES1	RAR responder		+			+	+
SERPING1	Ser (or Cys) clade G (C1 inhibitor (member 1)			+	+	+	+
SPP1	Secreted phosphoprotein 1	+	+	+	+	+	+
TGFβ	TGFβ superfamily proteins	+	+		+		+
Down-regulated							
CCNB	Cyclin B proteins	+	+	+			+
FRPHE	Frizzled related protein frpHE	+	+		+		+
GATA2	GATA-binding protein	+			+	+	
MSX1	Hox Msh-like protein 1	+		+		+	+
MSX2	Hox Msh-like protein 2	+	+	+			+
OLFM1	Olfactomedin related ER localised protein 1	+	+	+	+		+

Complement factor D (DF) is a serine protease essential for the activation of the alternative complement pathway and is expressed by macrophages whose numbers increase during the window of implantation (Lesavre *et al*, 1978).

Dickkopf-1 (DKK-1), a Wnt signalling antagonist, is a progesterone regulated gene which is thought to limit Wnt action during endometrial receptivity (Tulac *et al*, 2006), by binding low density lipoprotein (LDL) receptor like protein (Mao *et al*, 2001). Expression of DKK-1 is down-regulated during the window of implantation in women with endometriosis, which may have implication for the defective implantation associated with this condition (Kao *et al*, 2003).

IL-15 is an important endometrial chemokine, which mediates production of NK cells from stem or progenitor cells and also recruits CD16- NK cells into the endometrium from the peripheral blood (Keskin *et al*, 2007, Kitaya *et al*, 2005). In the absence of IL-15 in the null mouse model a lack of uNK cells recruitment is associated with a deficiency in spiral arteriole modification and placental development (Ashkar *et al*, 2003).

Progestagen associated endometrial protein (PAEP), also known as placental protein 14 or glycodelin, is elevated in the endometrium during the secretory phase of the menstrual cycle (Julkunen *et al*, 1986), where it represents one of the major glandular secretory products. PAEP is thought to be essential for implantation and is down-regulated in the presence of progesterone receptor antagonists which act as contraceptives (Uchida *et al*, 2007, Cameron *et al*, 1997).

The conceptus can regulate expression of secreted phosphoprotein 1 (SPP-1), which is also known as osteopontin, during decidualization (Herington *et al*, 2007). In the human endometrium SPP-1 appears to be regulated by progesterone (Apparao *et al*, 2001) and as a component of the ECM is suggested to be important for adhesion of the blastocyst to the luminal epithelium

Therefore, during the implantation window, gene array analysis has demonstrated elevation of a number of genes with relevance to implantation. However, it is intriguing that factors such as LIF, IL-11 or COX-2, which knockout studies have identified as important for

implantation, are not regulated in the endometrium at this time according to gene array analysis.

1.4.6.2. Reproductive phenotypes of knockout mouse models

By gene knockout models a number of factors, in addition to those discussed above, are suggested to be important in implantation. It is intriguing therefore that, given the apparent necessity of these genes in reproduction as indicated by their reproductive phenotypes, none of these genes appear to be changed in a consensus of gene array analyses described above. This may indicate that changes in these genes represent only small changes on gene arrays, which would be filtered out when selection criteria were applied.

Basigin is expressed in the luminal epithelium and the sub-luminal stroma specifically around the implanting blastocyst. However Basigin null mice fail to implant due to a maternal defect (Igakura *et al*, 1998, Kuno *et al*, 1998, Xiao *et al*, 2002a, 2002b).

FkBP52 (Fk506 binding protein 52) is a co-chaperone related to the heat shock protein family. FkBP52 null mice display compromised uterine progesterone receptor activity associated with aberrant expression of progesterone responsive genes such as HOXA-10, indian hedgehog, amphiregulin and histidine decarboxylase with a complete failure of implantation (Tranguch *et al*, 2005, Yang Z *et al*, 2006). FkBP52 therefore appears to be a critical determinant of progesterone actions in the uterus in preparation for implantation. As indicated in section 1.4.3, other heat shock family proteins have also been implicated in uterine receptivity and implantation.

As discussed in section 1.4.3, LIF, IL-6 and IL-11 signal through heterodimerisation of their receptors with gp130, with mice null for these factors or their specific receptor being infertile or displaying compromised implantation due to a maternal defect. The reproductive phenotype for the gp130 STAT null model is therefore unsurprising considering the apparent

requirement for these cytokines in implantation. The gp130 STAT null mouse displays no signs of implantation on day 5.5 post coitus (pc), the null mice however appeared to be fertile and transferred blastocysts developed in WT females. This suggests that downstream signalling of the IL-6 family members though heterodimerisation of its receptors is a necessary event for implantation, with a maternal defect responsible for the absence of implantation (Ernst *et al*, 2001). Indeed, the contraceptive mifepristone down-regulates signalling molecules which are induced by the IL-6 family via activation of gp130 (Catalano *et al*, 2003), it therefore appears that gp130 induced signalling is required for implantation.

HOXA-10 and -11 mice also appear to display implantation defects. HOXA-10 deficient mice appear to be unable to support decidualization and display a defect in uterine PGE_2 receptors, EP3 and EP4, and COX-2 expression (Benson *et al*, 1996, Hsieh-Li *et al*, 1995, Lim *et al*, 1999). The HOXA-11 null mice also display a complete absence of implantation, while WT mice can support the implantation and development of HOXA-11 deficient blastocysts (Hsieh-Li *et al*, 1995). These suggest a maternal defect is responsible for the implantation defect in both HOXA-10 and -11 mice.

Another homeobox gene, Hmx3, which belongs to a family distinct from the larger classes of homeobox genes, also displays an implantation defect with no implantation sites or decidual swellings on day 5.5 pc, however the implantation of Hmx3 null blastocysts into WT surrogates again suggests a maternal defect (Wang *et al*, 1998). These Hmx3 null mice also display an absence of uterine LIF expression on day 4 of pregnancy whish is essential for implantation (Stewart *et al*, 1992).

COX-2 signalling is essential for on-time implantation and decidualization (Lim *et al*, 1997, Cheng & Stewart, 2003). In rodents the predominant prostaglandin derived from COX-2 is prostacyclin (PGI_2), however, this is not the case in humans (Abel & Kelly, 1979). PGI_2 synthase and its nuclear receptor the Peroxisome proliferators activated receptor (PPAR)-δ, along with its obligate heterodimer the retinoid X receptor (RXR), are expressed at

the rodent implantation site along with COX-2 (Lim *et al*, 1999). PPARδ null animals display a placentation defect, which is characterised by the formation of only loose connections between the placenta and the maternal decidua (Barak *et al*, 2002), suggesting COX-2 - PGI_2 - PPARδ signalling to be involved in this function.

These data indicate potential roles for genes with known functions during the window of implantation and genes whose functions have still to be investigated. Consensus of genes expressed during the window of implantation from the gene array analysis may suggest genes which can be used as markers of receptivity as this is a hotly debated topic and thus far no-single marker has been implicated suggesting best practice may be to use a selection of markers. Data from gene array and gene knockout studies may also provide targets to examine in the case of infertility associated with a possible implantation defect, whether this problem represents unexplained infertility or infertility associated with luteal phase defect, endometriosis or PCOS. These molecular markers may be used in concert with the criteria for the morphological dating of the endometrium and represent a more accurate picture of the receptive state of the endometrium.

1.4.7. The effect of embryonic signals on the endometrium

It has been suggested that some changes which are necessary for implantation only occur in the event of pregnancy and are not observed in the luteal phase of non-pregnant cycles (Fazleabas & Strakova, 2002, Ghosh & Sengupta, 1998). This has been investigated in the mouse, *in vitro*, where it was observed that blastocysts attached to the endometrium from day 5 of pregnancy but did not attach in pseudopregnant mice where embryonic signals are absent (Shiotani *et al*, 1993). It therefore appears that the endometrium may not become fully receptive in the absence of specific signals from the pre-implantation embryo (Sharkey & Smith, 2003). HCG as one of the earliest embryonic products (Bonduelle *et al*, 1988, Lopata & Hay, 1989, Hay & Lopata, 1988) is a prime candidate to mediate these changes. Many studies have shown hCG to induce

morphological and secretory changes in the endometrium in addition to its action in preventing luteolysis of the corpus luteum (Shikone *et al*, 1996).

The LH/CG receptor is expressed in the non-pregnant endometrium and is localised to the glandular and luminal epithelium and stromal compartment (Bukovsky *et al*, 2003, Reshef *et al*, 1990, Licht *et al*, 2003, Bernadini *et al*, 1995). In the baboon, hCG treatment induces epithelial plaque formation in the luminal epithelium and induces decidual changes in stromal fibroblasts (Fazleabas *et al*, 1999) and hCG has also been shown to induce morphological and functional decidualization of human stromal cells (Han *et al*, 1999). Treatment with hCG of human endometrial cells *in vitro* and via a microdialysis system of human and baboon endometrium *in vivo* has demonstrated regulation of a number of growth factors and cytokines elevated during the window of implantation and implicated in implantation such as LIF, VEGF, M-CSF/CSF-1, MMP-9, PP14, IL-1, HB-EGF and IL-6 (Sherwin *et al*, 2007, Licht *et al*, 1998, 2001, 2007, Filicori *et al*, 2005, Perrier d'Hauterive *et al*, 2004, Uzumcu *et al*, 1998). A number of these factors are thought to have a reciprocal effect on the trophoblast cells in inducing syncytialisation and promoting/controlling invasion (Sawai *et al*, 1995a, 1995b, Ren *et al*, 1997, Morrish *et al*, 1998).

One of the first signs of successful implantation is an elevation of endometrial permeability, which is thought to be mediated by COX-2 and COX-2 derived prostaglandins. It has been demonstrated that hCG induces expression of COX-2 and production of PGE_2 in primary human stromal cells (Han *et al*, 1999, Zhou *et al*, 1999), suggesting that it is this embryonic signal, which mediates the endometrial permeability. It has also been shown that hCG can induce expression of proteins in leukocytes (Kosala *et al*, 2002) and peripheral blood mononuclear cells isolated from women in early pregnancy promote *in vitro* invasion of blastocysts when treated with hCG (Nakayama *et al*, 2002). The endometrial immune cells may release factors which modulate trophoblast invasion under the influence of hCG. The hormones to which the endometrium is exposed throughout the menstrual cycle and early pregnancy influence its development and preparation for pregnancy. Absence or dysregulation of one

or more of these factors may result in deficient endometrial preparation for implantation, a failure to implant or under- or over-invasion of the trophoblast resulting in pathological pregnancy conditions. In order to avoid these problems the hormonal influence on the endometrium must be tightly regulated throughout the cycle and early pregnancy.

1.5. The Prokineticins

The prokineticins are two pleiotropic proteins, which have predicted physiological and pathological functions in many tissues. Prokineticins were first described and named by Li *et al* (2001). Two cDNA's were identified which encoded two secreted proteins. These were shown to induce contraction in the smooth muscle of the gastrointestinal tract only and were designated PROK1 and prokineticin 2 (PROK2) due to this contractile activity. While this was the first paper to describe these proteins in the human, their expression in other species had been previously demonstrated.

PROK1 is the human orthologue of a non-toxic protein present in the venom of the black mamba snake *Dendroaspis polylepis* (Joubert & Strydom, 1980). This protein is known as venom protein A (VPRA) or mamba intestinal toxin 1 (MIT-1, Schweitz *et al*, 1990). This had previously been shown to induce contractions specifically in the ileum and distil colon of the guinea pig while conversely inducing relaxation in the proximal colon (Schweitz *et al*, 1999), which has also been demonstrated for the human prokineticins (Li M *et al*, 2001, Hoogerwerf, 2006). The contractile effect is mediated via voltage dependent calcium channels while the relaxation effect is mediated through nitric oxide release (Schweitz *et al*, 1999, Li M *et al*, 2001, Hoogerwerf, 2006). Subsequently LeCouter *et al* (2001) described a growth factor, which, similar to VEGF, induced a variety of effects in adrenal cortex endothelial (ACE) cells. These included a mitogenic response, chemotaxis, fenestration and angiogenesis and cyst formation in the ovary. This factor was designated endocrine gland vascular endothelial growth factor (EG-VEGF) due to its functional similarities with VEGF in ACE cells despite the absence of structural similarities (LeCouter *et al*, 2001). The amino acid sequences for EG-VEGF and PROK1 are identical thus differential functions were described for the same protein. Herein the proteins will be referred to as Prokineticins.

PROK2 is the human orthologue of a protein isolated from the skin of the yellow bellied frog *Bombina variegata*. This protein was designated Bv8 to reflect its origin (*Bombina variegata*) and its size of around 8kDa. Prior to its discovery in humans, expression of Bv8 had been reported in mouse testis, brain, ovary, kidney, uterus and heart (Wechselberger *et al*, 1999). Along with its homologue MIT-1, Bv8 has been reported to induce hyperalgesia in rats (Mollay *et al*, 1999). Prokineticins sensitise the transient receptor potential vanilloid 1 thus inducing hyperalgesia (Negri *et al*, 2002, Vellani *et al*, 2006).

PROK1 forms a mature protein of 86 amino acids with a signal peptide of 19 amino acids and PROK2 forms a mature protein of 81 amino acids (Li M *et al*, 2001, Lecouter *et al*, 2001). The gene encoding PROK1 is present on chromosome 1p21 (Kaser *et al*, 2003). PROK2 is the human paralogue of PROK1 and is located on chromosome 3p21.1 (Kaser, 2003). A splice variant of PROK2 has been identified in the testis of the human, mouse and bull. This splice variant contains an insert of 21 amino acids of which 11 are lysines or arginines; this led to the splice variant being named Bv8-basic (Wechelberger *et al*, 1999). The human PROK1 and PROK2 are around 58% identical (LeCouter *et al*, 2001). These proteins display significant homology with DKK-1 and Co-lipase (Glinka *et al*, 1998, Aravind *et al*, 1998). Interestingly the prokineticins, DKK-1 and Co-lipase share a common structural motif of 10 conserved cysteines which are predicted to form a fold within the molecule with disulphide bonding between the cysteines (van Tilbeurgh *et al*, 1992, Boisbouvier *et al*, 1998). A striking feature of the prokineticins is their N-terminal amino acid sequence - AVITGA, which is completely conserved. As may be expected by the conservation of this sequence, it is absolutely essential to the activity of these proteins. Mutations to this sequence produce prokineticin receptor antagonists or recombinant proteins, which have no effect at the receptors (Bullock *et al*, 2004, Negri *et al*, 2005).

1.5.1. Prokineticin receptors

The prokineticins are the cognate ligands for two closely related G-protein coupled receptors (GPCR's). In the study of Li *et al* (2001), which first described the human prokineticins, the receptors were suggested to be GPCR's due to the ability of GTPγ to displace prokineticin binding. The receptors were

subsequently further characterised and demonstrated to be GPCR's termed PROKR1 and PROKR2, these exhibit 85% amino acid identity with the greatest difference in their N-terminal sequences (Lin DC *et al*, 2002, Lin R *et al*, 2002, Masuda *et al*, 2002 *et al*, Soga, 2002). PROK1 preferentially activates PROKR1 while PROK2 preferentially activates PROKR2 (Lin DC *et al*, 2002). The prokineticin receptors are reported to couple to Gi (Lin R *et al*, 2002) Gq (Lin DC *et al*, 2002, Masuda *et al*, 2002, Soga *et al*, 2002) or Gs (Chen *et al*, 2005).

1.5.2. Tissue localisation and potential functions of the prokineticins

Expression of prokineticins and their receptors has been reported in a number of human and animal tissues including the testis (Wechelberger *et al*, 1999, Li M *et al*, 2001, LeCouter *et al*, 2001, Lin DC *et al*, 2002, Samson *et al*, 2004, Lecouter *et al*, 2003) prostate (Pasquali *et al*, 2006), ovary (Ferrara *et al*, 2003, Li M *et al*, 2001, Kisliouk *et al*, 2003, Fraser *et al*, 2005), placenta (LeCouter *et al*, 2001, Hoffmann *et al*, 2006, Chung *et al*, 2004, Li M *et al*, 2001), endometrium (Battersby *et al*, 2004a, Ngan, 2006 *et al*, Li M *et al*, 2001), and a number of non-reproductive tissues including the brain (Lin DC *et al*, 2002, Soga *et al*, 2002, Cheng *et al*, 2002, Ng *et al*, 2005, Li M *et al*, 2001) adrenal glands (Lecouter *et al*, 2001, Lin DC *et al*, 2002) and colon (Lin DC *et al*, 2002, Li M *et al*, 2001 *et al*, Soga, 2002). The expression of prokineticins in the colon is suggested to contribute to its contractility (Li M *et al*, 2001, Hoogerwerf, 2006). Expression of PROK2 in the brain and central nervous system is suggested to have a number of roles. PROK2 is suggested to have an essential role in supporting neuronal survival (Melchiorri *et al*, 2001). PROK2 exhibits a profile of circadian oscillation in the suprachiasmatic nucleus and is proposed to be responsible for the circadian locomotor rhythm (Cheng *et al*, 2002, Li JD *et al*, 2006). Studies both *in vitro* and *in vivo* have demonstrated the absolute necessity of PROK2-PROKR2 signalling in olfactory bulb neurogenesis and migration of gonadotropin releasing hormone (GnRH) neurones (Ng KL *et al*, 2005, Matsumoto *et al*, 2006). Mice lacking expression of PROKR2 display multiple defects including non-development of the olfactory bulb and atrophy of the male and female reproductive systems (Matsumoto *et al*, 2006).

A role for the prokineticins has also been suggested in the immune response and haematopoiesis. PROK2 is expressed in peripheral blood cells of the innate immune system including monocytes, dendritic cells and neutrophils, as well as bone marrow cells (Lecouter *et al*, 2004) while PROK1 is expressed in inflamed tissues (Dorsch *et al*, 2005). Prokineticins are proposed to promote survival and differentiation of granulocytic and monocytic cells and induce chemotaxis of macrophages with induction of an inflammatory phenotype (Martucci, 2005 *et al*, Dorsch *et al*, 2005). Expression of PROK2 and prokineticin receptors in progenitor and mature blood cells suggests a role for the prokineticins in haematopoiesis (LeCouter *et al*, 2004). In other tissues a role for PROK1 in angiogenesis associated with colorectal cancer and age related macular degeneration has been suggested in mouse models using *in vivo* PROK1 production (Goi, 2004 *et al*, Tanaka *et al*, 2006).

The prokineticins are expressed in male and female reproductive tissues. Their expression has been characterised in a number of tissues and pathologies, however their functions in these tissues have not yet been fully defined.

In the male reproductive tract, prokineticins and their receptors are expressed in the testis and the prostate. In the prostate the expression of PROK1 has been demonstrated in the hyperplastic and cancerous tissue (Pasquali *et al*, 2006) potentially suggesting a role for PROK1 in the pathogenesis of this disease. In the testis PROK1 localises mainly to the testosterone producing Leydig cells with PROK2 expression in the spermatocytes (Wechselberger *et al*, 1999, LeCouter *et al*, 2003, Samson *et al*, 2004). PROKR1 is expressed at higher levels than PROKR2 in the testis (Lecouter *et al*, 2003). Prokineticin signalling in the testis is proposed to modulate testosterone transport and angiogenesis in the normal tissue (LeCouter 2003 *et al*, Samson *et al*, 2004). Prokineticins also potentially contribute to angiogenesis in the pathological testis specifically in tumours of Leydig cells (Samson *et al*, 2004).

In female reproduction the prokineticins have potential roles in the normal functioning of the reproductive tract and pregnancy. In the normal ovary, expression of PROK1 is dynamic whereas PROK2 does not appear to be

expressed (Ferrara *et al*, 2003, Zhang *et al*, 2003). In the primordial and primary follicles PROK1 expression is demonstrated in the granulosa cells. In antral follicles, PROK1 expression is low in the theca cells with strong expression in the residual thecal cells of the mature atretic follicle (Ferrara *et al*, 2003). PROK1 expression in the extruded corpus luteum demonstrates elevation as it matures (Ferrara *et al*, 2003, Fraser *et al*, 2005). *In vitro* studies have suggested a role for PROK1 in angiogenesis in the corpus luteum though induction of VEGF expression and roles in proliferation and survival of corpora lutea endothelial cells (Kisliouk *et al*, 2005a, 2005b). In the polycystic ovary expression of PROK1 has demonstrated a correlation between ovarian hyperplasia and angiogenesis (Ferrara *et al*, 2003). Indeed, it has been demonstrated that delivery of PROK1 to the ovary results in gross angiogenesis and formation of ovarian cysts (LeCouter *et al*, 2001).

In the non-pregnant endometrium PROK1 is elevated during the secretory phase of the menstrual cycle and is specifically suggested to be elevated during the mid-secretory phase (Battersby *et al*, 2004a, Ngan *et al*, 2006, Figure 1.8). PROK2 and the prokineticin receptors are also expressed in the uterus but do not demonstrate temporal variation across the menstrual cycle (Battersby *et al*, 2004a). The prokineticins and their receptors localise to the epithelium, stromal and endothelial compartments of the endometrial functional layer in addition to the endothelial and smooth muscle cells of the myometrium (Battersby *et al*, 2004a). PROK1 expression is suggested to be down-regulated in endometrial cancer compared with the normal endometrium (Ngan *et al*, 2006) and is also suggested to disappear after menopause (Ngan *et al*, 2006). Expression of PROK1 is suggested to be modulated by ovarian steroid hormones; therefore cyclical expression of PROK1 may characterise endometrial development from menarche to menopause (Battersby *et al*, 2004a, Ngan *et al*, 2006, Figure 1.8). PROK1 may also have a role during the implantation window; PROK1 is elevated in the endometrium during the peri-implantation phase of the menstrual cycle when the endometrium becomes receptive to blastocyst implantation (Battersby *et al*, 2004a, Ngan *et al*, 2006). During this phase PROK1 may mediate endothelial leakage, one of the earliest signs of implantation (Plaks *et al*, 2006)

by inducing fenestrae formation and increasing microvascular permeability facilitating implantation (LeCouter *et al*, 2001).

PROK1 and PROKR1 are elevated in early placental tissues during the critical hypoxic period of placentation (weeks 8 - 10), with localisation of PROK1 to the syncytio- and cytotrophoblast (Hoffmann *et al*, 2006). PROK1 expression is inducible by hypoxia (LeCouter *et al*, 2001, Hoffmann *et al*, 2006) potentially suggesting a role for the prokineticins in placentation. It has been suggested that PROK1 may play a role in normal pregnancy but not in pathological pregnancy, as expression of PROK1 is not altered in pre-eclamptic placentas (Chung *et al*, 2004). PROK1, PROKR1 and PROKR2 have been localised and the pattern of expression investigated in the mouse placenta. PROK1 and PROKR1 are elevated from day 9.5 to day 10.5 pc in the mouse placenta, while PROKR2 is elevated from days 10.5 to 14.5 pc (Hoffmann *et al*, 2007). PROK1 is expressed in the labyrinth layer, with strong trophoblast staining, and the ectoplacental cone, while the receptors are expressed in the labyrinth layer, the trophoblast giant cells and the endothelial cells (Hoffmann *et al*, 2007). It is suggested that PROK1 may play dual roles within the mouse placenta via the trophoblast cells, contributing to placental growth, and via the giant cells, contributing to decidual invasion. As macrophages increase in number in the endometrium during early pregnancy and PROK1 induces chemotaxis of macrophages (Martucci *et al*, 2005) recruitment of macrophages may be another role for the prokineticins in early pregnancy.

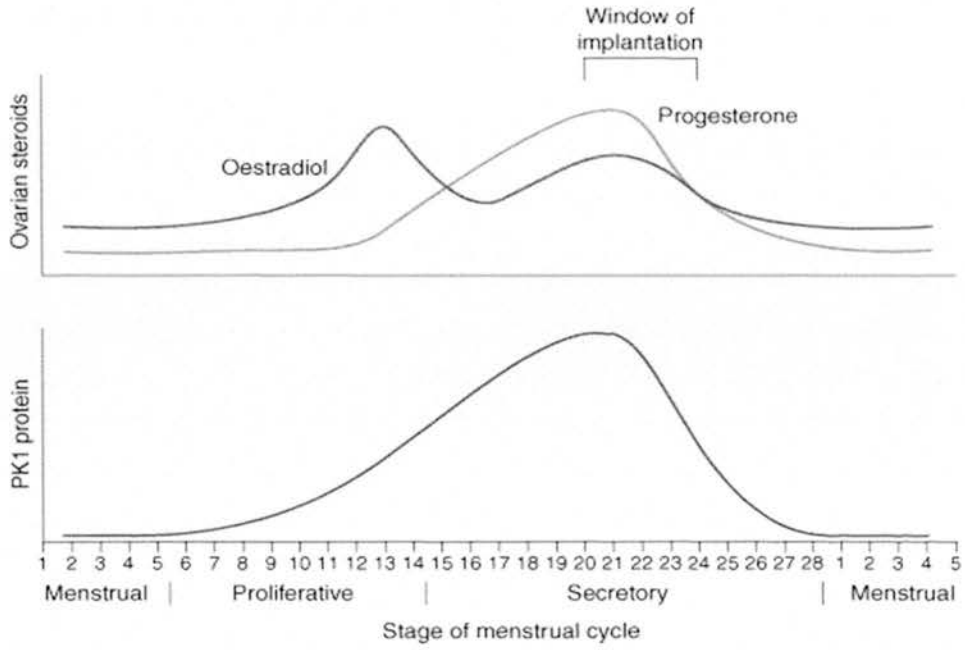


Figure 1.8. Representation of the dynamic variation in endometrial PROK1 expression in concert with Progesterone across the menstrual cycle. Adapted from Maldonado-Perez *et al*, 2007.

The prokineticins therefore have diverse proposed functions, which appear to be tissue specific. A great deal of clarification is required to delineate functions, signalling and gene activation in the various tissues. It appears that the main roles of PROK2 are in the brain while PROK1 appears to be mainly localised in the peripheral tissues.

1.5.3. Prokineticin 1 mediated signalling

Thus far, only preliminary signalling experiments have been performed in order to elucidate prokineticin mediated intracellular signalling. It has been established that prokineticin receptors are G-protein coupled receptors (Li *et al*, 2001, Lin R *et al*, 2002, Lin DC *et al*, 2002, Masuda *et al*, 2002, Soga *et al*, 2002, Chen *et al*, 2005) however, their G-protein coupling has not thus far been established. Additionally, the main body of work investigating prokineticin mediated intracellular signalling has been performed on immortalised cell lines transfected with prokineticin receptors with little thought given to the potential differences in signalling inherent in different tissues or different pathological states. As indicated above, the study of Li *et al* predicted the prokineticin receptors to be GPCR's due to the ability of GTPγ to displace prokineticin binding (Li *et al*, 2001). In 2002 a number of papers were published describing

the G-protein coupling of the prokineticin receptors, however, differences in signalling emerged. In the paper of Lin R *et al* (2002), prokineticin receptors were reported to be Gi coupled, activate ERK 1/2, Akt and Enos phosphorylation and was not dependent on VEGF signalling (a schematic representing Gi coupled receptor mediated signalling is indicated in Figure 1.9). Gi mediated intracellular signalling leads to inhibition of cAMP production and can result in ERK 1/2 phosphorylation.

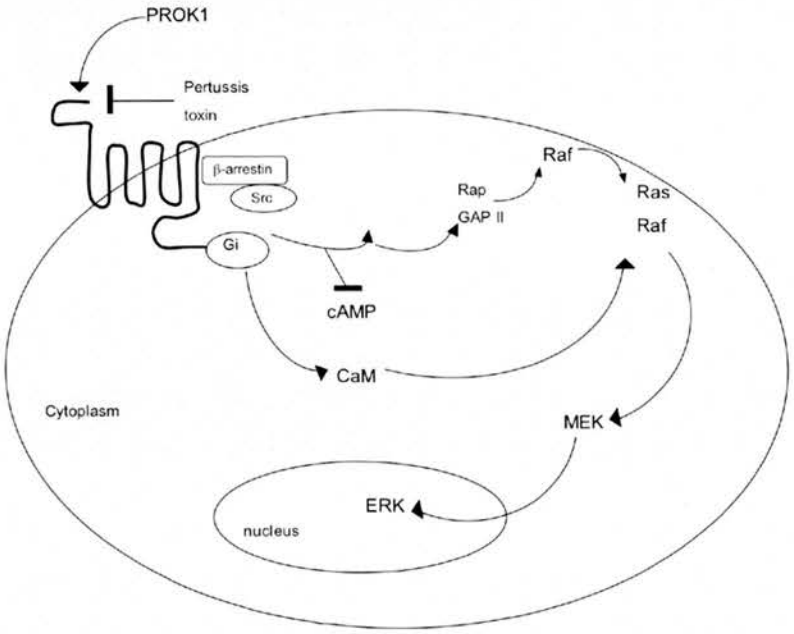


Figure 1.9. Schematic representing Gi coupled receptor signalling

The work of R Lin *et al* was performed in primary cells, namely bovine adrenal cortex endothelial cells. Other reports published on prokineticin mediated signalling, however, disagreed with the indication that the prokineticin receptors were Gi coupled. The reports of Lin DC *et al* (2002), Masuda *et al* (2002) and Soga *et al* (2002), suggested prokineticin receptors to be Gq coupled. These studies examined established second messengers induced upon Gq-coupled receptor activation, including calcium and inositol phosphate mobilisation (a schematic representing Gq coupled receptor mediated signalling is indicated in Figure 1.10). Additionally, Chen *et al* (2005) reported that stimulation of cells expressing prokineticin receptors 1 and 2 with prokineticin 2 induced production of c AMP and activation of the CRE (c AMP response element) promoter, therefore suggesting the receptors to be Gs

coupled (schematic representing Gs coupled receptor mediated signalling is indicated in Figure 1.11) The studies, however, were not performed in primary cells but immortalised HEK293 (human embryonic kidney), COS-7 (African green monkey kidney fibroblast) and CHO (Chinese hamster ovary) cells transfected with the prokineticin receptors. This allowed one ligand-one receptor studies to be performed as these cells can be maintained in culture with stable expression of the transfected receptors. Whereas primary cells, which have a limited culture time, may express different receptors and different levels of receptors, therefore it is difficult to know which receptors and how much of each receptor is expressed without PCR and protein analysis of receptor expression in each batch of cells. However, an inerrent problem with immortalised transfected cells is that they may not represent the actual physiological situation. The current reports examining prokineticin mediated signalling indicate that prokineticin receptors can couple to multiple G-proteins, and thus the signalling outcome may depend on which receptor - ligand combination is present. Thus far, no examination of the signalling mediated via prokineticin receptors has been performed in a specific human tissue or pathological condition.

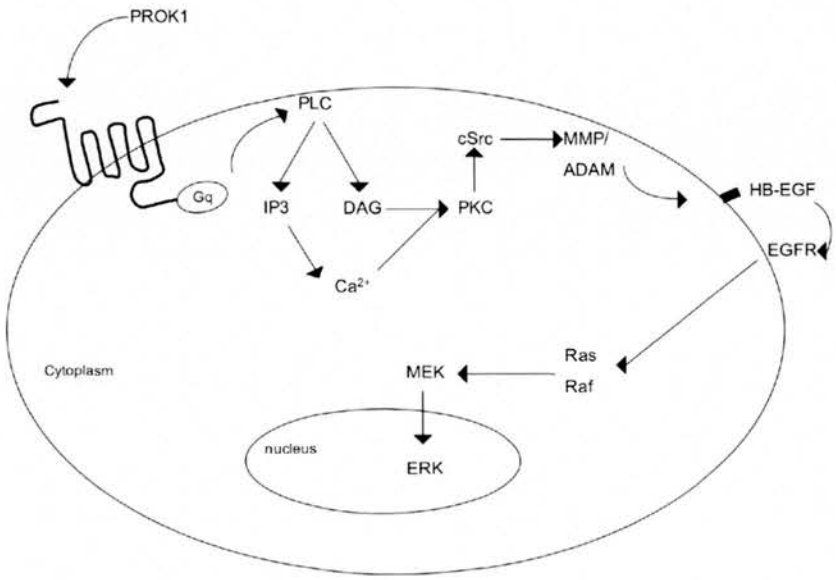


Figure 1.10. Schematic representing Gq coupled receptor signalling

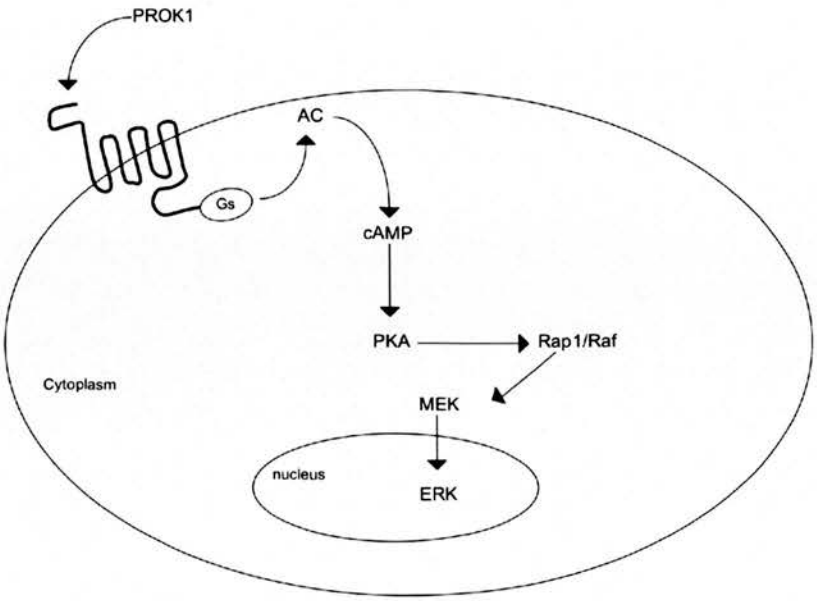


Figure 1.11. Schematic representing Gs coupled receptor signalling

1.6. Aims and objectives of this thesis

As mentioned in section 1.4, an inadequacy in uterine receptivity accounts for a growing proportion of implantation failures and is now considered as a major cause of infertility in otherwise healthy women (Achache & Revel, 2006). Infertility is a growing problem with a great socio-economic impact on health-care and resources. Assisted conception programmes are high cost, both emotionally and financially for the people involved. Much effort has been directed to identifying human markers of uterine receptivity. However, no outstanding candidate has thus far emerged. Uterine receptivity may therefore be defined by a number of histological, hormonal, morphological and molecular parameters. Elevation of PROK1 during the secretory phase of the menstrual cycle revealed a protein with potential as a marker of uterine receptivity. However, to date, little is known about PROK1 and its role in the endometrium. It was therefore hypothesised that endometrial PROK1 signalling via PROKR1 may contribute to uterine receptivity and possibly the implantation process. The specific aims of this research were to: -

1. Further examine the expression and localisation of PROK1 and PROKR1 in the non-pregnant endometrium and determine the expression and localisation of these factors in the pregnant and pathological endometrium. This was conducted on endometrial tissue taken across the menstrual cycle, from 7 - 12 weeks of gestation and from well, moderately and poorly differentiated endometrial cancer samples.

2. Characterise an endometrial cell line stably expressing PROKR1 and determine the signalling pathways activated upon PROK1-PROKR1 interaction.

3. Determine PROK1 target genes activated upon PROK1-PROKR1 interaction. This was investigated by gene array analysis using the Affymetrix GeneChip® Human Genome U133 Plus 2.0 and the ABI 1700 v.2 Applied Biosystems Human Genome Survey microarrays.

4. Further investigate the temporal regulation and mechanism of two PROK1 regulated genes, COX-2 and LIF.

5. Determine the physiological relevance of the data obtained using the PROKR1 Ishikawa cell line. This was investigated by protein phosphorylation cascade activation, temporal regulation and mechanism of gene expression induced by PROK1 in first trimester decidua.

6. Determine potential paracrine mediators of PROK1 expression in early pregnancy. This was investigated by examination of PROK1 and LIF expression induced by hCG in PROKR1 Ishikawa cells and first trimester decidua

Chapter 2 - General materials and methods

2.1. Reagents and suppliers

A list of suppliers is provided in appendix 1

2.2. Tissue collection

2.2.1. Endometrial Tissue

Endometrial tissue collection was performed with ethical approval from Lothian Research Ethics Committee under ethics number LREC/05/S1103/32. Written informed consent was obtained from all subjects prior to tissue collection. Endometrial biopsies at different stages of the menstrual cycle were obtained from women with regular menstrual cycles (25 - 35 days) who had not received a hormonal preparation in the 3 months preceding biopsy collection. Samples were collected either with an endometrial suction curette (Pipelle, Laboratoire CCD) or as full thickness endometrial biopsies (including functional layer and basal-myometrial junction) from women undergoing hysterectomy for benign gynecological conditions. Shortly after collection, tissue was snap frozen in dry ice or placed in RNAlater (Ambion) and stored at -70°C (for RNA extraction) or fixed in neutral buffered formalin and wax embedded (for immunohistochemical analysis). Biopsies were dated according to stated last menstrual period and confirmed by histological assessment by a pathologist. Furthermore, circulating estradiol and progesterone concentrations (assay described in Section 2.9.3) at the time of biopsy were consistent for both stated last menstrual period and histological assignment of menstrual cycle stage. Samples were divided according to phase of menstrual cycle as early proliferative, mid proliferative, late proliferative to ovulatory, early secretory, mid secretory and late secretory

2.2.2. Decidua tissue

Decidua tissue collection was performed with ethical approval from Lothian Research Ethics Committee under ethics number LREC/05/S1103/32. Written informed consent was obtained from all subjects prior to tissue collection. First trimester decidua (7 - 12 weeks) was collected from women undergoing elective first trimester surgical termination of pregnancy (STOP).

Shortly after collection, tissue was placed in RNAlater (Ambion) and stored at -70°C (for RNA extraction), fixed in neutral buffered formalin and wax embedded (for immunohistochemical analysis) or placed in RPMI 1640 (Sigma; containing 2mM (mmol/litre) L-glutamine, 100U penicillin, and 100μg/ml streptomycin, PAA laboratories) for tissue culture. Gestation of pregnancy was confirmed by ultrasound scan prior to procedure.

2.2.3. Endometrial cancer tissue

Endometrial cancer tissue collection was performed with ethical approval from Lothian Research Ethics Committee under ethics number LREC/1999/6/4. Written informed consent was obtained from all subjects prior to tissue collection. Endometrial cancer tissue was obtained from women undergoing surgery for removal of endometrial cancer. Shortly after collection, tissue was placed in RNAlater (Ambion) and stored at -70°C (for RNA extraction) or fixed in neutral buffered formalin and wax embedded (for immunohistochemical analysis). Cancer biopsies were assessed by a pathologist and assigned a grade, grade 1 - well differentiated, grade 2 - moderately differentiated or grade 3 - poorly differentiated.

2.3. Cell culture

2.3.1. PROKR1 receptor transfection and amplification

This cell line was kindly produced by Dr K Morgan. Human cDNA encoding prokineticin receptor 1 (PROKR1) was prepared using RT-PCR. RNA from IMR-32 neuroblastoma cells was reverse transcribed using random primers (Promega) and superscript reverse transcriptase (Invitrogen). Receptor cDNA was amplified using Easy-A DNA polymerase (Stratagene) and specific primers:

Forward 5′ GGA TCC AAG CTT GAT GGA GAC CAC CAT GGG G 3′
Reverse 5′ CTC GAG GAT ATC TTA TTT TAG TCT GAT GCA GTC CAC CT 3′.

The PCR product was excised from a 1% agarose gel following electrophoresis, purified, ligated into sequencing vector pCR4 (Invitrogen) and cloned into

E.Coli TOP10 cells. Cloned plasmid DNA was subjected to automated DNA sequencing to confirm the cDNA sequence prior to subcloning the Hind III-EcoRV fragment into pcDNA 3.1(+). This expression construct was transfected into Ishikawa cells using electroporation and G418 resistant clones were isolated. These cells are subsequently termed PROKR1 Ishikawa cells.

2.3.2. Cell culture conditions

A cell line of immortalised endometrial cells (Ishikawa) was used throughout the study. The Ishikawa cell line was deposited by A. Taylor at the University of Leicester from endometrial epithelial cells taken from endometrial adenocarcinoma. Cells were transfected with PROKR1 cDNA in the sense direction (as indicated in Section 2.3.1) and cultured in complete DMEM Glutamax medium (Section 2.11) supplemented with 200µg/ml G418 antibiotic. All cell culture took place in humidified conditions at 37°C with 5% carbon dioxide. Cells were routinely passaged in T162 flasks at 37°C to maintain sufficient numbers of cells for experimental treatments. Wild type (WT) Ishikawa cells were maintained as above without the addition of G418 antibiotic.

2.3.2.1. Passaging of cells

Cells grown in T162 flasks were passaged at 3-4 day intervals, when cell density within culture vessels reached ~70%. Complete DMEM Glutamax medium was removed and cells were washed twice with approximately 10ml phosphate buffered saline (PBS), without $CaCl_2$ or $MgCl_2$, to remove any remaining medium containing serum. The PBS was removed and replaced with 3ml of 0.05% Trypsin-EDTA and cells incubated for approximately 5 minutes at 37°C until the cells became detached from the surface of the culture flask. Trypsin digestion was stopped by addition of 7ml Complete DMEM Glutamax medium containing serum, sufficient to inactivate trypsin activity. In order to passage the cells, 2ml of the cell suspension was transferred to a fresh T162 flask and the total volume of the Complete DMEM Glutamax medium made up to 25ml. Cells to be seeded out for experimental use were counted using a haemocytometer and plated into culture vessels at the density indicated in Table 2.

Table 2. Cell density in culture vessels

Culture vessel	Cell density (cells/compartment)
6-welled plate	2.5×10^5
12 welled plate	1×10^5
10cm dish	3×10^6
6cm dish	5×10^5
3cm dish	2×10^5

2.3.3. Cell and tissue treatments

PROKR1 Ishikawa cells were treated with PROK1 (Promokine, reconstituted in water) at various concentrations (4pM, 40pM, 400pM, 4nM, 40nM) in order to determine the optimal dose of PROK1 to be used in subsequent studies (the physiological concentration of PROK1 is currently unknown). 40nM was revealed as the dose of PROK1 that elicited the optimal response in PROKR1 Ishikawa cells, and was therefore used in all subsequent studies in PROKR1 Ishikawa cells and first trimester decidua tissue.

In order to determine the maximal time of PROK1 induced ERK 1/2 phosphorylation, PROKR1 Ishikawa cells or first trimester decidua were treated with 40nM PROK1 for 0, 1, 5, 10, 20, 30, 45 or 60 minutes.

In order to determine the time of maximal gene expression in response to PROK1, PROKR1 Ishikawa cells or first trimester decidua were treated with 40nM PROK1 for 0, 2, 4, 6, 8, 12 or 24 hours.

In order to study signalling molecules activated by PROK1-PROKR1 in signalling to ERK 1/2, specific inhibitors of signalling molecules were used. Similarly, in order to determine the signalling molecules that lead to PROK1 mediated gene expression specific inhibitors of signalling molecules were used.

Indicated in Table 3 is a list of the inhibitors used, the signalling molecules they specifically inhibit and the concentration at which they were applied to the PROKR1 Ishikawa cells or first trimester decidua tissue. Actinomycin D, an inhibitor of transcriptional activation and Cyclohexamide, an inhibitor of protein synthesis were also used. All inhibitors were used at concentrations at

or below their IC50 or working concentrations reported for other systems and were dissolved in DMSO. YM254890 was a kind gift of Dr Jun Takasaki; all other inhibitors were purchased from Calbiochem.

Table 3. Concentrations of inhibitors

Inhibitor	Molecules inhibited	Inhibitor concentration	IC50/working concentration
YM254890	Gq protein	1μM	1μM
Pertussis toxin	Gi protein	200ng/ml	1μg/ml
U73122	Phospholipase C-β	10μM	10nM
BAPTA-AM	Calcium chelator	50μM	2mM
PP2	cSrc	10μM	50μM
AG1478	EGFR	200nM	100μM
PD98059	MEK	50μM	50μM
NS-398	COX-2	10μM	11.3mM
Cyclohexamide	Protein synthesis	10μg/ml	100μg/ml
Actinomycin D	Transcriptional activity	250ng/ml	5μg/ml

In order to understand how the various inhibitors inhibit intermediate signalling molecules the mechanism of inhibition mediated by these chemicals must be understood.

YM254890 is a cyclic peptide isolated from the culture broth of Chromobacterium SP (Taniguchi *et al*, 2003). It contains uncommon amino acids β-hydroxyleucine, N,O- dimethylthreonine and N-methyldehydroalanine (Taniguchi *et al*, 2003). YM254890 blocks the exchange step of GDP for GTP in $G_{\alpha q}$ activation. YM254890 inhibits Ca2+ mobilisation in cells specifically expressing Gq coupled receptors. Constitutively active Gq R183C is completely inhibited by YM254890. This is a fairly new inhibitor and the half life is currently unknown.

The mechanism of action of U73122 is thought to involve the electrophillic maleimide side chains of the molecule, as substitution of this moiety results in loss of activity (Smith RJ, 1990). It is suggested that the inhibition occurs by alkylation of sulfhydryl groups or other nuceophiles needed for the activation or activity of a protein permissive for second messenger function. The steroid

portion of the molecule is necessary for the inhibitory action in part by conferring lipophilicity to the molecule (Yule, 1992). U73122 inhibits intracellular calcium mobilisation at the level of IP3 production (Smith *et al*, 1990). Additionally, GTPγS stimulation of phosphoinositide turnover is inhibited by U73122 (Yule *et al*, 1992), demonstrating that U73122 acts at the level of PLC. The half life of U73122 in cell culture systems is unclear as it has recently been demonstrated that the availability of U73122 to interact with PLC may be hampered through conjunction of the malemide group to non-specific biological nucleophiles such as L-glutamine, reducing the biological half life of the molecule (Wilsher NE, 2007)

BAPTA-AM is the cell permeable form of BAPTA. It is hydrolysed once loaded into cells by Cytosolic esterases and is trapped intracellularly as the active chelator BAPTA (Calbiochem). Chelation is the binding or complexation of a bi- or multi-dentate ligand, in this case calcium. BAPTA-AM sequesters calcium rendering it unable to initiate downstream signalling events.

Upon expression of Src family members $p56^{lck}$ and $p59^{fynT}$ by vaccinia virus, PP2 specifically inhibited their activity. PP2 also inhibits the phosphorylation of endolase, this is specifically mediated by Src family member $p56^{lck}$ (Hanke *et al*, 1996)

EGFR forms a ternary complex with ATP and the peptide substrate. AG1478 competes with ATP binding to cause signalling inhibition (Ward *et al*, 1994). This occurs due to the non-polar meta- substituents on the aniline ring of the molecule (Wakeling *et al*, 1996). It is therefore competetitive with ATP and non-competitive with substrate.

MEK's are activated by phosphorylation on serine residues by up-stream kinases. MEK's can then catalyse phosphorylation of both threonine and tyrosine residues. PD98059 blocks tyrosine and threonine phosphorylation of MAPKK. PD98059 does not compete for ATP binding and inhibits through an allosteric mechanism, PD98059 does not bind the active site but interacts at another site thereby blocking access to activating enzymes. PD98059 actively

inhibits activation and phosphorylation of MEK (Dudley *et al*, 1995, Alessi *et al*, 1995).

NS-398 acts as a competitive, non-reversible inhibitor specifically for COX-2 over COX-1. NS-398 binding to COX-2 mediates a conformational transition of the enzyme leading to significantly tighter binding of the inhibitor to the enzyme without any chemical modification of the enzyme or inhibitor. NS-398 competitively binds to the active site of COX-2 only as COX-1 mediated prostaglandin production is not inhibited in the presence of NS-398 (Futaki *et al*, 1994, Copeland *et al*, 1994).

Actinomycin D inhibits RNA polymerase by complexing with DNA via deoxyguanosine residues (Calbiochem)

Cyclohexamide inhibits transferase II (Felicetti *et al*, 1966), this action is sulfhydryl dependent (Sutler *et al*, 1966). Cyclohexamide may retard the peptide elongation by inactivating the sulfhydryl groups of transferase II. The GTP hydrolysis reaction required for chain elongation also involved sulfhydryl dependent enzymes (Baliga *et al*, 1969).

Human chorionic gonadotropin (hCG, Sigma) was used at a concentration of 1IU (international unit). The physiological concentration appears to vary from around 1IU to 33000 IU, varying between patients, gestation of pregnancy etc. Therefore 1IU of hCG was chosen as the minimum dose likely to elicit a response in both cells and tissue. Previous studies have used doses ranging from 1-50IU (Perrier d'Hauterive, 2004).

2.3.4. Transient transfection of c-Myc ERK, dominant negative isoforms of signalling molecules and COX-2 luciferase promoter reporter constructs

2.3.4.1. Production and characterisation of dominant negative isoforms of signalling molecules

The dominant negative constructs utilised in this study were kindly provided by Dr Zvi Naor (Department of Biochemistry, Tel Aviv

University, Tel Aviv, Israel) and have been previously characterized and described (Harris *et al*, 2002, Levi *et al*, 1998). Further information on the production and characterisation of DN-cSrc, DN-EGFR, DN-Ras and DN-MEK are indicated below.

DN-cSrc was originally cloned by Nada *et al* (1991), it is also known as Csk. Csk is a protein tyrosine kinase, which negatively regulates cSrc by specifically phosphorylating a negative regulatory site. Initially Csk was purified from the membrane fraction of neonatal rat brain by sequential column chromatography. The cDNA that encodes Csk was cloned and the primary structure deduced (nucleotide sequence and deduced amino acid sequence provided in Figure 2.1, Nada *et al*, 1991). Csk lacks a tyrosine residue at a site corresponding to tyrosine 416 - a site for autophosphorylation. The members of the Src family share a highly conserved sequence around this site and are autophosphorylated at a tyrosine residue equivalent to Tyr416. Csk however shows no autophosphorylation. The dominant negative effect was demonstrated by Nada *et al* (1991) and Dikisc *et al* (1996).

DN-EGFR was originally cloned by Livneh *et al* (1986). Full length c DNA was pieced together from overlapping λ phage clones λ HER-A21 and subcloned into PBR322. Final subclone was extended fromn the Sst I site, 15bp before the initiation codon for translation. Both ends were changed to Xho I sites using synthetic DNA linkers and subcloned into the Pvu II site of PBR 322 that was changed to an Xho I site in a similar manner. EGFR receptor c DNA with Xho I ends was placed into and early substitution SV40 based expression vector. The Sma I site was changed to an Xho I site and was used for cloning the plasmid. The final plasmid in the correct orienttarion is denoted as pLSX. Plasmid pL△ B71 was constructed by partial digestion of pLSX with Bal I at nucleotide 2485. Xba I inkers were added and excess linkers removed by digestion with Xba I followed by agarose gel electrophoresis. Plasmid pL△ NA8 (DN-EGFR) was constructed from pL△ B71 . pL△ B71 was digested by Nar I and

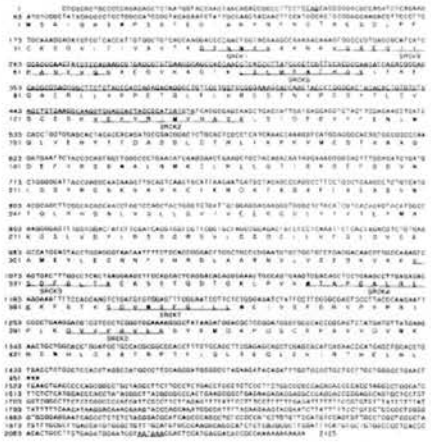
staggered ends filled in with a Klenow fragment of DNA polymerase I. The blunt ended fragment was ligated with a synthetic 26-bp double stranded oligonucleotide composed of 18bp which represents sequence 2199 - 2217 of the EGF receptor attached to a 7bp sequence containing the Xba I site, with the sequence 5′ - CCA CAT CGT TCG GAA GCC TCT AGA G - 3′. The ligated fragment was cleaved by Sma I and excess Xba I was purified by gel electrophoresis. This 1.2kb fragment was further ligated to a 8kb Sma-Xba I fragment which was recovered from p LSX. The activity of pL△ NA8 as a dominant negative was demonstrated by Livneh *et al* (1986) and Kashles *et al* (1991). The plasmid map for DN-EGFR is provided in Figure 2.1.

The Asn-17ras^{H}p21 (DN-Ras) gene was isolated by Feig *et al* (1988) by randomly mutagenising a cellular ras^{H} bacterial expression vector (p XCR) and screening for GTP binding mutants with a bacterial colony GTP binding assay. The entire ras-coding sequence of the metant was determined by the dideoxy method (Sanger *et al*, 1977). This construct has preferential affinity for GDP (Feig *et al*, 1988). The Asn-17 mutant was isolated as a ras^{H} mutant with preferential affinity for GDP versus GTP. The asparagines for serine substitution at position 17 maps to a region of p21 that is highly conserved among all GTP-binding proteins. The dominant inhibitory action of this construct is demonstrated by Feig *et al* (1988) and Stacey *et al* (1991). Asn-17ras^{H}p21 displayed approximately a 40 fold decrease in affinity for GTP binding compared with normal rasHp21 with GDP binding maintained.

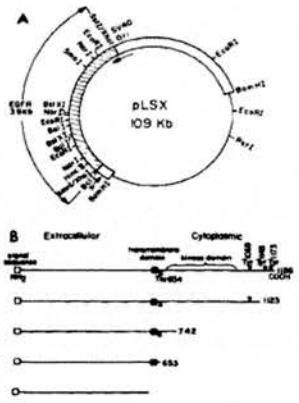
In order to produce DN-MEK, MKK1a (MAP kinase kinase/MEK) cDNA was modified to ensure optial overexpression by generating a Kozak sequence in the 5′ end of the c DNA, The oligonucleotide GGA TCC GCC GCC ACC ATG GCA CCC AAG AAG AAG CCG was used in a PCR reaction to replace the first 55 base pairs of MKK1a (Seger *et al*, 1992). The product was then inserted into Eco RI and Bam HI sites of the pc DNA 3 vector DNA which contains a gene for neomycin resistance, propagated in XL-1 cells and sequenced to ensure it correst composition. Point mutations

were made by PCR using the above construct as a template for K97A (DN-MEK), the sense primer is TCA TGG CCA GAG CGC TAA TTC ATC and the antisense primer is GGA GTT GGC CAT GGA AGT CGG T. Mutations were sequenced to ensure their correct composition. Seger *et al* (1994) demonstrate that K97A acts as a dominant inhibitory molecule as it is an inactive form of MEK. The site of MAPK mutations is indicated in Figure 2.1.

A



B



C

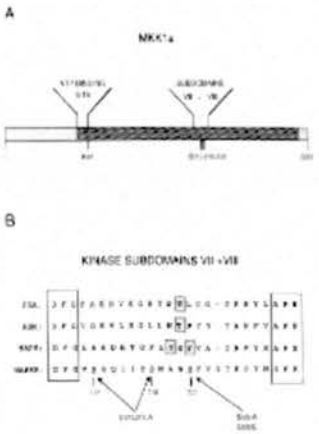


Figure 2.1. Dominant negative constructs for A. DN-cSrc, B. DN-EGFR and C. DN-MEK.

2.3.4.2. Transient transfection of c-Myc ERK and dominant negative isoforms of signalling molecules

In order to investigate the signalling molecules involved in PROK1 mediated signalling to ERK 1/2, PROKR1 Ishikawa cells were co-transfected with c-Myc tagged ERK and dominant negative isoforms of

signalling molecules or empty vector (pcDNA3). PROKR1 Ishikawa cells were seeded into 6 cm dishes at the density indicated in Table 2. 2.5μg of c-Myc ERK was co-transfected into PROKR1 Ishikawa cells with 7.5μg of dominant negative (DN) isoforms of signalling molecules in the presence of Superfect transfection reagent (Qiagen) for 6 hours. The transfection media was removed and replaced with complete DMEM Glutamax (without G418) for at least 24 hours. Subsequently the PROKR1 Ishikawa cells were serum starved overnight for a minimum of 16 hours in serum free DMEM Glutamax medium prior to stimulation with 40nM PROK1.

2.3.4.3.Transient transfection of COX-2 luciferase promoter reporter constructs

In order to investigate the COX-2 promoter activation induced by PROK1, PROKR1 Ishikawa cells were transfected with the full-length COX-2 luciferase promoter reporter construct. PROKR1 Ishikawa cells were seeded into 12 well plates at the density indicated in Table 2. The COX-2 promoter reporter construct linked to firefly luciferase (1.5μg) was co-transfected into PROKR1 Ishikawa cells with an internal control, pRL-TK (0.15μg, containing the renilla luciferase coding sequence, Promega) for 6 hours in the presence of Superfect transfection reagent (Qiagen). The COX-2 promoter reporter plasmid consisting of a 966-bp fragment of the COX-2 promoter (C2.1; -917 to +49) ligated to a firefly luciferase construct as described by Bradbury *et al* (2003), was kindly donated by Dr Robert Newton (Biomedical research Institute, Department of Biological Sciences, The University of Warwick, Warwick, UK). The transfection media was removed and replaced with complete DMEM Glutamax (without G418) for at least 24 hours. Subsequently the PROKR1 Ishikawa cells were serum starved overnight for a minimum of 16 hours in serum free DMEM Glutamax medium. In order to investigate the temporal regulation of PROK1 mediated COX-2 promoter activation, transfected PROKR1 Ishikawa cells were treated with 40nM PROK1 for 0, 2, 4, 6 and 8 hours. In order to examine the signaling pathway activation that leads to PROK1 mediated COX-2 promoter activation, PROKR1 Ishikawa cells were treated with specific inhibitors of Gq, PLC-β, Ca^{2+}, cSrc, EGFR and MEK at the

concentration indicated in Table 3 for 1 hour, prior to treatment with 40nM PROK1 for 6 hours. Luciferase promoter reporter activity was determined by luciferase assay as described in Section 2.4.

2.3.4.4. Transient transfection of COX-2 luciferase promoter reporter constructs and dominant negative isoforms of signalling molecules.

In order to further investigate the signalling molecules that lead to PROK1 induced COX-2 promoter reporter activation, PROKR1 Ishikawa cells were co-transfected with the COX-2 luciferase promoter reporter construct and renilla, as indicated in Section 2.3.4.2, and 1.5μg of DN isoforms of signalling molecules or empty vector. The transfection was performed as indicated in Section 2.3.4.2. The transfection media was removed and replaced with complete DMEM Glutamax (without G418) for at least 24 hours, subsequently the PROKR1 Ishikawa cells were serum starved overnight for a minimum of 16 hours in serum free DMEM Glutamax medium. Transfected cells were subsequently treated with 40nM PROK1 for 6 hours. Luciferase promoter reporter activity was determined by luciferase assay as described in Section 2.4.

2.4. Luciferase promoter reporter assay

The activity of both firefly and renilla was determined using the dual luciferase assay kit (Promega) and total luciferase activity was determined by dividing the relative light units generated by the firefly luciferase by relative light units generated by the renilla luciferase in the same reaction. Fold increase in luciferase activity was calculated by dividing the total luciferase activity in cells treated with PROK1 by the total luciferase activity in cells treated with vehicle or transfected with empty vector.

2.5. Polymerase chain reaction

2.5.1. RNA extraction and quantification

RNA was extracted from both cells and tissue using TRI-reagent (total RNA isolation reagent). TRI-reagent was used in conjunction with heavy gel phase lock tubes. The protocol used was a modified phenol - chloroform extraction. Samples in TRI-reagent were loaded into phase lock tubes and Bromo-chloro

propane added to the solution, the tubes were then shaken vigorously for at least 15 seconds in order to allow adequate mixing of the two solutions. Samples were then allowed to rest for approximately 10 minutes to allow the two phases (organic and inorganic) to begin to separate. The samples were then centrifuged at 4°C for 15 minutes at 14000 RPM to ensure complete separation of the two phases. Phase lock tubes form a wax layer between the two phases allowing the top aqueous layer to be simply poured into a fresh tube without risk of DNA or protein contamination. This aqueous layer is retained and mixed with isopropanol in order to precipitate the RNA present in solution. Samples were again centrifuged at 14000 RPM for 15 minutes at 4°C in order to pellet the precipitated RNA present in the solution. RNA pellets were subsequently washed in 70% RNase free ethanol, before being resuspended in RNase free water.

Lysis and homogenisation of cells was achieved by addition of TRI-reagent and mixing by pipetting to ensure a homogenous sample preparation for RNA isolation. Lysis and homogenisation of tissue samples was achieved by addition of TRI-reagent followed by homogenisation with a tissue lyser (Qiagen). Tissue samples were snap frozen in 2ml eppendorf tubes, which contained a 5mm steel bead. After addition of TRI-reagent the tubes were loaded onto the homogeniser and subjected to shaking at 25Hz for 6 minutes. This allowed the tissue to be homogenised by the action of the steel bead. The homogenised tissue samples were centrifuged at 4°C for 10 minutes at 15000 RPM to clear any cellular debris. After homogenisation samples were loaded into heavy gel phase lock tubes (Eppendorf) and processed as above. All tubes used in processing of cells and tissues were RNase free and tips used were RNase free filter tips. Benches and other equipment were treated with RNase Zap (Ambion) to eliminate RNase activity.

RNA concentrations were determined using the Genequant spectrophotometer or the Nanodrop ND-1000 spectrophotometer. The Genequant and Nanodrop spectrophotometers were used to determine nucleic acid concentration and purity from a 1:10 diluted 10µl sample (Genequant) or a neat 1µl sample (Nanodrop). Light absorbance of the sample was measured at wavelengths of

260nm and 280nm. The absorbance at 260nm indicates the RNA concentration, and the 260:280 absorbance ratio indicates the quality of the sample. A pure sample of RNA should have a 260:280 ratio in the region of 1.9. A 260:280 ratio lower than this indicates contamination of the sample by protein. The samples were compared against a blank standard consisting of RNase free water, which was used to set zero for the OD measurements. After samples had been quantified a final reading of water was taken to ensure adequate cleaning of equipment had been performed between sample readings.

2.5.2. Reverse transcriptase polymerase chain reaction

Reverse transcriptase PCR was performed on cDNA prepared from total RNA isolated from cultured cells in an oligo dT primed reaction. cDNA for each reaction was synthesised from 1μg RNA in a volume of 10μl. Briefly, cDNA was prepared as follows: -

1μg RNA in 10μl volume
1μl Random Hexamers (200ng/μl)
1μl d NTP (10μM)

This mix was incubated at 65°C for 5 minutes and then placed on ice. Subsequently:

4μl 1st strand buffer
2μl DTT
1μl RNase inhibitor

were added and the mix and incubated at 37°C for 2 minutes, followed by addition of:

1μl Superscript reverse transcriptase enzyme.

The mix was then incubated at: -

37°C for 50 minutes
70°C for 15 minutes

In order to amplify PROKR1 or PROK1 cDNA, PCR was performed using specific primers. The sequences are given in Table 4.

Table 4. Primer sequences for RT-PCR

PROKR1 forward	5′ GGA TCC AGG CTT GAT GGA GAC CAC CAT GGG G 3′
PROKR1 reverse	5′ CTC GAG GAT ATC TTT TAG TCT GAT GCA GTC CAC CT 3′
PROK1 forward	5′ GTG CCA CCC GGG CAG 3′
PROK1 reverse	5′ AGC AAG GAC AGG TGT GGT GC 3′

The PCR reaction proceeded as follows. Primers were diluted 1/10 from a stock concentration of 100pM to 10pM prior to use in the PCR reaction. A mastermix was prepared consisting of:

10x buffer	2µl
$MgCl_2$	1.2µl
dNTP's	0.4µl
Forward primer	0.4µl
Reverse primer	0.4µl
Taq enzyme	0.25µl
cDNA	2 µl
H_20	to 20µl

This mix was placed in a Touchdown PCR machine under the following conditions:

95°C	10 minutes	
94°C	30 seconds	Repeated x 40
65°C	30 seconds	
72°C	1 minute 30 seconds	
72°C	10 minutes	
4°C	hold	

A 1% Tris-Acetate-EDTA (TAE) agarose gel containing ethidium bromide was then prepared and samples electrophoresed for approximately 1 hour 30 minutes at 100v before visualisation using a UV transilluminator.

2.5.3. Taqman quantitative PCR

Expression of mRNA within tissue and cultured cells was examined by Taqman PCR in order to quantify expression and detect low expression transcripts. In order to perform this quantitative method cDNA was prepared

in a random hexamer primed reaction using 200ng of RNA per reaction. A reaction mix was prepared as indicated below for each cDNA synthesis reaction. All PCR reagents were purchased from Applied Biosystems.

RNA (100ng/μl)	2μl
10x RT buffer	1μl
25mM Magnesium Chloride	2.2μl
10mM dNTP's	2μl
Random Hexamers	0.5μl
RNase inhibitors	0.2μl
Multiscript Reverse Transcriptase	0.25μl
RNase free water	1.85μl

Reaction mixture was incubated at:

25°C	90 minutes
48°C	45 minutes
95°C	5 minutes

Quantitative PCR was conducted on the cDNA using specific primer-probe mixtures designed and optimised for the detection of the gene of interest in human. Primer-probe combinations specific to PROK1, PROKR1, COX-2, LIF, IL6, IL-8 or IL-11 were designed in-house and were custom synthesised and purchased from Eurogentec. Sequences of primers and probes are given in Table 5. The probe was fluorescently labelled with FAM at the 5′ end with a TAMRA quencher dye at the 3′ end. In the presence of the gene of interest, the probe anneals to the sequence of interest between the forward and reverse primers. The 5′ 3′ nuclease activity of the Taq DNA polymerase cleaves the probe and separation of the reporter dye from the quencher results in a measurable increase in fluorescence. An increase in fluorescence is indicative of an elevation in the PCR product of interest. Gene expression was normalised by including primer-probe mix to detect the Vic labelled 18s ribosomal subunit as a loading control for the amount of cDNA added in each sample. The 18s primer and probe sequences are given in Table 5. Each Taqman PCR reaction (in duplicate) consisted of:

cDNA	2µl
RNase free water	19.05µl
Mastermix	25µl
Forward primer	0.6µl
Reverse primer	0.6µl
Probe	2µl
18s (forward, reverse, probe mix)	0.75µl

Reaction mixtures were loaded, in duplicate, each consisting of 20µl onto a 96-well MicroAmp fast optical reaction plate (Applied Biosystems) for analysis on an ABI 7900 HT Fast Real-Time PCR machine (Applied Biosystems). Data were analysed and processed using Sequence detector version 1.6.3 (Applied Biosystems) according to the manufacturers instructions. Results were expressed as relative to an internal positive RNA standard (cDNA obtained from a single sample of endometrial tissue) included in all reactions. The data was analysed utilising the comparative C_T method for relative quantification. Briefly, the amount of target is normalised to an endogenous control and relative to a calibrator using the formula: -

$$2^{-\Delta\Delta CT}$$

Table 5. Taqman primer and probe sequences

Gene	Primers and probe
PROK1 forward	5′ GTG CCA CCC GGG CAG 3′
PROK1 reverse	5′ AGC AAG GAC AGG TGT GGT GC 3′
PROK1 probe (FAM)	5′ACA AGG TCC CCT TGT TCA GGA AAC GCA 3′
PROKR1 forward	5′ TCT TAC AAT GGC GGT AAG TCC A 3′
PROKR1 reverse	5′ CTC TTC GGT GGC AGG CAT 3′
PROKR1 probe (FAM)	5′ TGC AGA CCT GGA CCT CAA GAC AAT TGG 3′
COX-2 forward	5′ CCT TCC TCC TGT GCC TGA TG 3′
COX-2 reverse	5′ ACA ATC TCA TTT GAA TCA GGA AGC T 3′
COX-2 probe (FAM)	5′ TGC CCG ACT CCC TTG GGT GTC A 3′
LIF forward	5′ TGG TGG AGC TGT ACC GCA TA 3′
LIF reverse	5′ TGG TCC CGG GTG ATG TTG 3′
LIF probe (FAM)	5′ TCG TGT ACC TTG GCA CCT CCC TGG 3′
IL-8 forward	5′ CTG GCC GTG GCT CTC TT 3′
IL-8 reverse	5′ TTA GCA CTC CTT GGC AAA ACT G 3′
IL-8 probe (FAM)	5′ CCT TCC TGA TTT CTG CAG CTC TGT GTG AA 3′
IL-6 forward	5′ GCC GCC CCA CAC AGA CA 3′
IL-6 reverse	5′ CCG TCG AGG ATG TAC GGA AT 3′
IL-6 probe (FAM)	5′ CCA CTC ACC TCT TCA GAA CGA ATT GAC AAA C 3′
IL-11 forward	5′ CCC AGT TAC CCA AGC ATC CA 3′
IL-11 reverse	5′ AGA CAG AGA ACA GGG AAT TAA ATG TGT 3′
IL-11 probe (FAM)	5′ CCC CAG CTC TCA GAC AAA TCG CCC 3′
18s forward	5′ CGG CTA CCA CAT CCA AGG AA 3′
18s reverse	5′ GCT GGA ATT ACC GCG GCT 3′
18s probe (VIC)	5′ TGC TGG CAC CAG ACT TGC CCT C 3′

2.6. Inositol phosphate assay

Cells were seeded in 12-well plates in complete DMEM Glutamax for 24 hours prior to incubation with inositol free special DMEM (Sigma) containing 1μCi/ml [^{3}H] myo-inositol (Amersham) for 24 hours. Cells were washed sequentially with buffer A and buffer A containing 10nM LiCl, before incubation with PROK1 at a range of concentrations (indicated in Section 2.3) for 60 minutes. The agonist induced inositol phosphate response is amplified in the presence of Li^+ (Berridge *et al*, 1982), therefore addition of LiCl enables cleaner results to be obtained. Reactions were terminated by the removal of PROK1 stimulus and cells were permeabilised by the addition of ice-cold 10mM formic acid. These samples were incubated at 4°C for approximately 60 minutes prior to loading of supernatants into tubes containing AG 1-X8 resin. In order to separate total tritiated inositol phosphate from formic acid the ion exchange method of Berridge (1983) was employed. Sample loaded resin was washed sequentially with water and 60mM

ammonium formate/5mM sodium tetraborate with resin vortexed and allowed to settle before removal of the supernatant with care taken not to disturb the settled resin. Subsequently bound tritiated myo-inositol was eluted from the resin by the addition of 1M ammonium formate/0.1M formic acid. 2.5ml of scintillation fluid was added to the samples and the tritium content of the eluted fractions measured by liquid scintillation counting in a Beckman 5000 scintillation counter, counted with an automatic quench correction for one minute per sample.

2.7. Western immunoblot analysis

2.7.1. Protein extraction and quantification

2.7.1.1.Cells

Following stimulation, cells were washed twice in ice cold PBS without $CaCl_2$ and $MgCl_2$ followed by addition of NP40 lysis buffer. Cells were incubated in NP40 lysis buffer on ice for approximately 30 minutes in order for the cells to lyse. Cells were harvested using a rubber policeman and transferred to a fresh eppendorf tube. Cell lysates were cleared of cell debris by centrifuging at 4°C for 15 minutes at 15000 RPM. Cleared lysates were transferred to fresh eppendorf tubes, diluted 1:50 and quantified using a modified Bradford protein assay kit (BioRad Laboratories). Protein concentrations were calculated using Assay Zap software and a standard curve generated by BSA standards of known concentrations.

2.7.1.2.Tissue

Decidua tissue explants were finely chopped using sterile scissors prior to treatment. Following stimulation, tissue samples were snap frozen in 2ml eppendorf tubes, which contained a 5mm steel bead. NP40 lysis buffer was added to the tubes and proteins harvested by homogenisation following the tissue homogenisation protocol as indicated in Section 2.5.1. Briefly, samples were loaded onto a tissue lyser (Qiagen) and shaken for 6 minutes at 25Hz. Tissue lysates were cleared of cell debris by centrifugation, as indicated above in Section 2.7.1.1 and quantified using protein assay kits (BioRad Laboratories) as indicated above in Section 2.7.1.1.

2.7.2. Immunoprecipitation

Following stimulation, cells ($5x10^5$ for c-myc ERK experiments, $3x10^6$ for cSrc and EGFR experiments) were washed twice in ice cold PBS without $CaCl_2$ and $MgCl_2$ followed by addition of NP40 lysis buffer. Cells were incubated in NP40 lysis buffer on ice for approximately 30 minutes in order for the cells to lyse. Cells were harvested using a rubber policeman and transferred to a fresh eppendorf tube. Cell lysates were cleared of cell debris by centrifuging at 4°C for 15 minutes at 15000 RPM. Cleared lysates were transferred to fresh eppendorf tubes and equal amounts of protein incubated with specific p-Tyr 418 (for investigation of temporal regulation of cSrc and EGFR, Santa Cruz) or c-myc (for investigation of the effect of dominant negative isoforms of signaling molecules on PROK1 induced ERK 1/2 phosphorylation, Santa Cruz) antibody preconjugated to protein A-Sepharose beads. Cell lysates were incubated with preconjugated antibody overnight at 4°C with gentle rotation. The beads were spun down at 4°C for 15 minutes at 15000 RPM, the supernatant removed, fresh NP40 lysis buffer added and the beads resuspended. This was repeated in order to wash the beads thoroughly. Excess lysis buffer was removed with a Hamilton syringe after the last wash and immune complexes solubilized in 20µl Laemmli buffer [125mmol/L Tris-HCl, 4% SDS, 5% 2-Mercaptoethanol, 20% glycerol, 0.05% Bromophenol blue].

2.7.3. Western immunoblot analysis

In order to examine the effect of the dose of PROK1 on ERK 1/2 phosphorylation (dose response), and the time course and phosphorylation cascade leading to ERK 1/2 phosphorylation in PROKR1 Ishikawa cells, 20µg of protein was resuspended with Laemmli buffer. In order to examine the time course of PROK1 mediated ERK 1/2 and phosphorylation cascade leading to ERK 1/2 phosphorylation in first trimester decidua, 50µg of protein was resuspended in Laemmli buffer. For COX-2 expression, 40µg of protein was resuspended in Laemmli buffer. In order to examine the effect of dominant negative isoforms of signaling molecules on PROK1 induced ERK 1/2 phosphorylation and examine the temporal regulation of cSrc and EGFR by PROK1 samples were prepared by immunoprecipitation as indicated above.

Proteins were denatured by boiling for 5 minutes and resolved on a 4 - 20% SDS-PAGE gradient gel (Invitrogen) run in parallel with SeeBlue protein loading standards (Invitogen) at 40 milliamps for approximately 2 hours, amount of protein loaded is indicated above. Proteins separated on the gel were then immunoblotted to a PVDF membrane (Millipore). The Polyvinylidene difluoride (PVDF) membrane was sequentially submerged in methanol, water and then transfer buffer. Blotting paper was also submerged in transfer buffer. A 'sandwich' was constructed comprising blotting paper, PVDF membrane, and SDS-PAGE gel followed by a final layer of blotting paper. In this order the blotting 'sandwich' was placed in a semi-dry blotting apparatus (Bio-Rad) and proteins transferred by application of 14 volts for 90 minutes. Non-specific binding of antibody was prevented by incubation of the immunoblot with blocking buffer, chemifluorescence probed immunoblots were blocked with 4% bovine serum albumin (BSA), fluorescent probed immunoblots were blocked with a low-fluorescence blocking solution (LiCor). The immunoblots were incubated with specific primary antibodies for phosphorylated ERK 1/2, total ERK 1/2, phosphorylated cSrc, phosphorylated EGFR, COX-2 and β-actin (antibody dilutions indicated in Table 6) and detected either by alkaline phosphatase-conjugated secondary antibodies, for chemifluorescent detection, or fluorescent labeled secondary antibodies, for direct fluorescent detection

Table 6. Antibody dilutions used for Western immunoblots detection

Antibody	Species raised in	Dilution	Purchased from
pERK	Rabbit	1:1000	Cell Signalling
tERK	Mouse	1:1000	Cell signalling
COX-2	Goat	1:500	Santa Cruz/ Autogen Bioclear
B-Actin	Goat	1:800	Santa Cruz/ Autogen Bioclear
p-EGFR	Goat	1:500	Santa Cruz/ Autogen Bioclear
cSrc	Rabbit	1:500	Santa Cruz/ Autogen Bioclear

2.7.3.1.Chemifluorescent detection

As indicated above, alkaline phosphatase-conjugated secondary antibodies were used for ECF - chemifluorescent detection of antibody bound proteins on the immunoblot. The immunoblots were incubated with primary

antibody, in 4% BSA blocking buffer, overnight at 4°C with gentle motion. The immunoblots were then washed a number to times in TNS-tween before exposure to an alkaline phosphatase conjugated secondary antibody, directed against the species in which the primary antibody was raised. The antibody was left to incubate and bind to the immunoblots for one hour at room temperature with gentle motion. The immunoblots were again washed with tris neutral saline (TNS)-tween and then exposed to ECF chemifluorescent substrate (Amersham) for 20 minutes in order to visualise immunoreactive proteins. Immunoblots were kept in the dark for this period. Subsequently, excess ECF was removed from the immunoblot and proteins revealed by phosphorimager analysis using the Typhoon 9400 phosphorimager system. A schematic of the ECF detection process described is shown in Figure 2.1. In order to control for protein loading, the blots were stripped, washed in TNS and the process repeated with an antibody to control for loading e.g. total ERK 1/2. Protein expression was quantified with Imagequant TL v2003.03, and relative density in the immunoblots calculated by dividing the value obtained from the phosphorylated immunoblots by the value obtained from the loading control blots and expressed as fold above vehicle treated control.

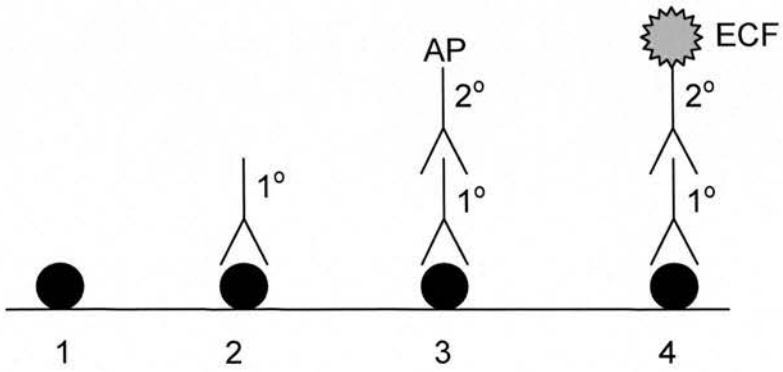


Figure 2.2. Schematic representing the ECF Western immunoblot detection method.

1. Proteins separated in the SDS-PAGE gel are immunoblotted from the gel to the PVDF membrane by use of a Transblot semi-dry transfer cell (BioRad) run at 14V for 90 minutes. The PVDF membrane is subsequently blocked with 4% BSA blocking buffer for 60 minutes.

2. The primary antibody is applied to the immunoblot in 4% BSA blocking buffer. The antibody is left to bind to the protein of interest overnight at 4°C.
3. The immunoblot is subsequently washed in TNS-tween and the secondary antibody, with an alkaline phosphatase label, is applied in 4% BSA blocking buffer for one hour.
4. The ECF substrate is subsequently applied to the immunoblot for 20 minutes. The ECF reacts with the alkaline phosphatase enzyme present on the secondary antibody and gives off a chemifluorescent signal. The immunoblot is scanned and the chemifluorescent signal detected. The immunoblots are subsequently stripped of the antibody and probed with an antibody to detect a loading control.

2.7.3.2.Direct fluorescent detection

Immunoreactive proteins were visualised by a direct fluorescent system. In contrast to the above chemifluorescent system, both the phosphorylated protein or main protein of interest and the total/loading control protein could be detected at the same time. This can be achieved by using primary antibodies raised in different species. These can then be visualised by using fluorescently labelled secondary antibodies directed against the different species, which emit at different wavelengths. This has a number of advantages; (a) there is no danger of over stripping the blot and thus losing some of the protein of interest and, (b) provided the immunoblots are kept in the dark, the fluorescence will not fade enabling the immunoblots to be re-scanned if required.

The immunoblots were incubated overnight at 4°C with primary antibodies in low fluorescence blocking buffer with gentle motion, e.g. for detection of phosphorylated and total ERK 1/2, the immunoblots were incubated with rabbit anti pERK 1/2 and mouse anti tERK 1/2. The immunoblots were washed a number of times with PBS-tween before exposure to the fluorescently labelled secondary antibodies e.g. goat anti rabbit antibody which emits at 680nm (to detect pERK) and goat anti mouse antibody which emits at 800nm (to detect tERK). The antibody was left to bind to the immunoblots for one hour at room temperature in black boxes in order

to avoid bleaching of the fluorescent label. The immunoblots were then washed a number of times in PBS-tween before visualisation of the proteins on the LiCor fluorescent scanner set to detect the different wavelengths emitted. These different wavelengths were visualised with different colours (680nm - red, 800nm - green) in order to visually differentiate between the two. A schematic of the process is described in Figure 2.2. Proteins were quantified using the LiCor software. Relative density in immunoblots was calculated by dividing the value obtained from the phosphorylated blots by the value obtained from the total blots and expressed as fold above vehicle controls.

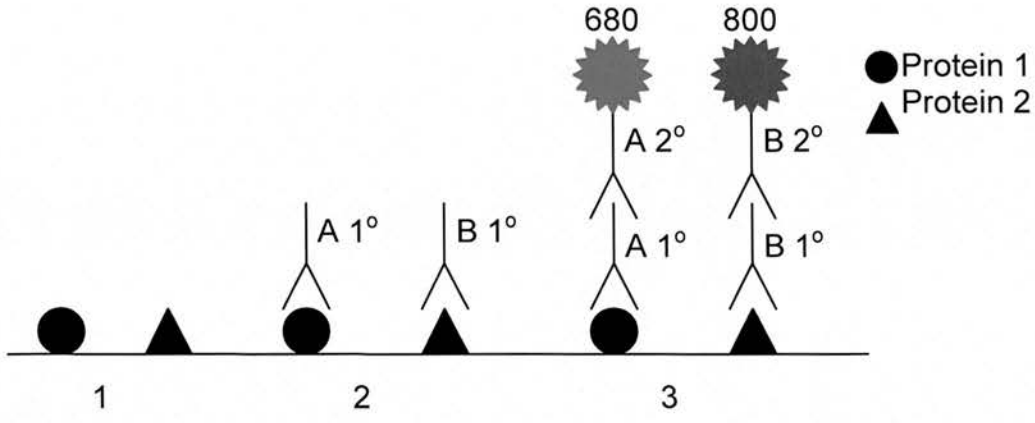


Figure 2.3. Schematic representing fluorescent Western immunoblot detection method

1. Proteins separated in the SDS-PAGE gel are immunoblotted from the gel to the PVDF membrane by use of a Transblot semi-dry transfer cell run at 14V for 90 minutes. The PVDF membrane is subsequently blocked with low fluorescence blocking buffer for 60 minutes.
2. The proteins of interest are detected with antibodies raised in two different species, e.g. in order to detect pERK, anti-rabbit pERK is used (protein 1), in order to detect tERK, anti mouse tERK is used (protein 2). The antibody is left to bind to the protein of interest overnight at 4°C.
3. The immunoblots are subsequently washed in PBS-tween. The primary antibodies are detected with fluorescently labelled secondary antibodies raised against the different species. These antibodies have fluorescent labels, which emit at either 680nm (red, protein 1) or 800 nm (green,

protein 2). The immunoblots are incubated with the secondary antibodies for one hour and the immunoblots scanned with a fluorescent scanner (LiCor).

2.8. Immunohistochemistry

2.8.1. Tissue fixation, wax embedding and Sectioning

Tissues were fixed in 4% NBF (neutral buffered formaldehyde) for 24 hours and then transferred to 70% ethanol. Tissues were subsequently processed and wax embedded using the Leica TP 1050 processor as summarised in Table 7.

Table 7. Tissue processing protocol.

	Station	Time	Temperature	Pressure/vacuum
Ethanol 70%	1	1 hr 30 minutes	Ambient	Ambient
Ethanol 80%	2	1 hr 30 minutes	Ambient	Ambient
Ethanol 90%	3	1 hr 30 minutes	Ambient	Ambient
Ethanol 95%	4	1 hr 30 minutes	Ambient	Ambient
Ethanol 95%	5	1 hr 30 minutes	Ambient	Ambient
Abs. Ethanol	6	2hrs	Ambient	Ambient
Abs. Ethanol	7	2 hrs	Ambient	Ambient
Xylene	8	1 hr	Ambient	Ambient
Xylene	9	1 hr	Ambient	Ambient
Xylene	10	1 hr	Ambient	Ambient
Paraffin wax	Left	1 hr	60 degrees	P/V
Paraffin wax	Middle	1 hr	60 degrees	P/V
Paraffin wax	right	1 hr 30 minutes	60 degrees	P/V

Wax embedded Sections were chilled on ice prior to being cut into 5μm sections and floated in warm water. Sections were then floated onto glass slides before being baked overnight.

2.8.2. 3,3'-diaminobenzidine (DAB) immunohistochemistry

In order to examine protein localization of PROK1 or PROKR1 in endometrium or first trimester decidua, immunohistochemical analysis was performed on tissue sections prepared as indicated in Section 2.6.1. Tissue sections (5μM) were dewaxed in xylene, sequentially rehydrated in decreasing concentrations of ethanol (100%, 95%, 80%, 75%) and finally placed in water when

rehydration was complete. Antigen retrieval was performed in order to reveal the epitope sites of the proteins and make them available for the antibodies to bind. This was achieved by placing the rehydrated tissue sections in a pressure cooker and boiling them for five minutes in 0.1% citrate buffer (pH 6.0). The sections were allowed to rest and cool for 20 minutes before placing the slides in tap water. Endogenous peroxidase activity was quenched with 3% (vol/vol) H_2O_2 in methanol at room temperature. Blocking serum (5% serum in Tris-buffered saline with 0.05% BSA) was applied for one hour in order to block any non-specific binding. Subsequently PROK1 or PROKR1 antibodies (dilutions given in Table 8) were applied in the serum block solution and incubated overnight at 4°C. The tissue sections were then washed sequentially in TNS-tween and TNS to remove any excess antibody before application of the secondary antibody, a biotinylated labelled antibody directed against the species in which the primary antibody was raised. In order to detect the antibody binding, and therefore protein expression, an avidin-biotin peroxidase detection system was employed. The sections were washed in TNS and exposed to the avidin biotin complex (ABC) reagent (Dako) for 30 minutes. The sections were once again washed in TNS before exposure to the chromagen 3,3"-diaminobenzidine, the colour development was checked under a microscope and the reaction terminated by placing the slides in water. The slides were counterstained with haematoxylin, dehydrated in increasing concentrations of alcohol and mounted.

Table 8. Antibody concentrations used in DAB immunohistochemistry detection

Antibody	Species raised in	Dilution	Purchased from
PROK1	Rabbit	1:1000	Phoenix pharmaceuticals
PROKR1	Rabbit	1:250	Lifespan Biosciences

2.8.3. Double immunofluorescent histochemistry

Co-localizations were performed as described in individual chapters as the protocol varies depending on the combination of antibodies used. Briefly, Tissue sections (5μM) were dewaxed in xylene, sequentially rehydrated in decreasing concentrations of ethanol (100%, 95%, 80%, 75%) and finally placed in water when rehydration was complete. Antigen retrieval was performed in order to reveal the epitope sites of the proteins and make them available for

the antibodies to bind. This was achieved by placing the rehydrated tissue sections in a pressure cooker and boiling them for five minutes in 0.1% citrate buffer (pH 6.0). The sections were allowed to rest and cool for 20 minutes before placing the slides in tap water. Endogenous peroxidase activity was quenched with 3% (vol/vol) H_2O_2 in methanol at room temperature. Blocking serum (5% serum in phosphate buffered saline with 0.05% BSA) was applied for one hour in order to block any non-specific binding. Subsequently the antibody was applied in the serum block solution and incubated overnight at 4°C. The tissue sections were then washed sequentially in PBS-tween and PBS to remove excess antibody. A biotinylated antibody was applied for one hour followed by a streptavidin detection system. All subsequent steps were performed in the dark in order to avoid bleaching of the fluorescent detection labels. Sections were subsequently washed in PBS-tween/PBS followed by re-blocking in blocking serum and incubation with the second detection antibody overnight at 4°C. The following day the sections were washed in PBS-tween/PBS followed by application of a biotinylated or peroxidase antibody and a streptavidin or tyramide detection system. Sections were washed in PBS and incubated with nuclear counterstain ToPro (1:2000) in PBS. Sections were mounted in Permafluor, coverslipped, and fluorescent images were visualised using a laser scanning confocal microscope (LSM 510, Carl Zeiss, Jena, Germany). In this work, negative control sections were incubated with IgG from the appropriate species ie. for sections in which rabbit primary antibodies were used, rabbit IgG was applied. Sections were subsequently treated as all other sections with secondary antibodies and detection reagents applied. This should indicate non-specific binding of the primary antibody. However, it should also be considered that the secondary antibody may bind non-specifically. The secondary antibodies are raised against IgG of the species in which the primary antibody is raised, however, the discrimination between IgG's of different species (eg. Differences between IgG from rabbit vs goat) may be doubted. Therefore, after application of the first primary antibody the secondary antibody directs against the second primary antibody should be applied. However, the second blocking step applied at the end detection of the first primary antibody should eliminate this possibility.

2.9. Gene array

2.9.1. Sample preparation

PROKR1 Ishikawa cells were seeded in complete DMEM Glutamax medium in 5 x 6cm dishes for PROK1 treated and vehicle treated samples and allowed to settle for 24 hours. The following day cells were incubated in serum free DMEM Glutamax medium for at least 16 hours. Subsequently, the cells were stimulated with 40nM PROK1 or vehicle for 8 hours. RNA was extracted using RNeasy extraction kits (Qiagen). The principle of which is the binding of RNA strands, from lysed and homogenised samples, to a silica membrane in the presence of a high salt buffer and ethanol. Contaminants within the samples are removed through a series of three washes, and a purified RNA sample is finally eluted from the membrane in RNase free water, achieved through centrifugation. In order to extract the RNA, cells were lysed in guanidine-thiocyanate buffer and removed from the plates using rubber policemen (cell scrapers). The cells were immediately homogenized. The lysis and homogenisation of the cells was achieved by passing the lysate through a 0.22 bore syringe a number of times in the presence of highly denaturing guanidine-thiocyanate buffer to prevent degradation by RNases.

RNA samples were initially quantified using the Genequant spectrophotometer, to give an initial indication of their concentration and quality. Samples were then diluted to a concentration, which allowed further quantification and quality assessment on the 2100 Bioanalyser and RNA nanochip system (Agilent Technologies). The criteria set for sample inclusion in the gene array was a 260/280> 1.9. This was assessed by the genequant, the quality, as indicated, was further assessed with the RNA nanochip system, this gave an RIN (RNA integrity number) which should be around 2, and a value for 18s/28s, which should also be around 2. Only samples which passed these quality control tests were included in the pooled samples. The 5 plates from each treatment were pooled to create one pooled RNA sample. This was done to reduce sample variability. This experimental process was performed 3 times in total to produce 3 PROK1 treated and 3 vehicle treated RNA samples. These were hybridized on 6 AB1700 and 6 Affymetrix arrays as follows.

Hybridisation of the RNA samples to the gene arrays was performed commercially by Geneservice.

2.9.1.1. ABI

RT-IVT was carried out in accordance with the Applied Biosystems Chemiluminescent RT-IVT nanoamp (one-cycle) labeling protocol. The samples were fragmented and subsequently prepared for hybridization to Applied Biosystems Human Genome Survey Microarray, version 2, for 16 hours. Following hybridization, the arrays were stained, washed using the Applied Biosystems Chemiluninescence detection kit and scanned using the Applied Biosystems 1700 Chemiluminescent Microarray Analyser.

2.9.1.2. Affymetrix.

cDNA synthesis and Biotin labeling of cRNA were carried out using the Ambion Message Amp II cRNA kit. The samples were subsequently fragmented, prepared for hybridisation using the Affymetrix hybridisation control kit and hybridised to GeneChip® Human Genome U133 plus 2.0 arrays for 16 hours. Following hybridization the GeneChip arrays were stained, washed on the fluidics station and scanned.

2.9.2. Data analysis

This analysis was performed by Geneservice. Data acquired using the ABI technology were filtered according to the manufacturer recommendations (AB1700 Data Analysis, Applied Biosystems, 2005). All probes with the Flags measurement of more than 100 and S/N ratios less than 3 in more than 2 out of 6 samples were discarded. This resulted in a set of high-quality probes for further processing. Data acquired using Affymetrix technology were generated using the GCOS version 1.3 software package. After the pre-processing steps, the data for both platforms were normalized using vsn, variance stabilizing normalization by W. Huber (Huber, 2002). Normalized data were analyzed for differential expression with the LIMMA package by G.K. Smyth (Smyth, 2004). Probe-wise linear models were fitted and then computed moderated t-statistics and log-odds of differential expression by empirical Bayes shrinkage of the standard errors towards a common value. The p-values for the contrast

between treated and untreated samples were adjusted for multiple testing with Benjamini and Hochberg method (Benjamini & Hochberg, 1995).

Cross mapping between AB1700 probes and Affymetrix probesets was courtesy provided by Applied Biosystems. All unmapped poorly annotated genes were removed from the analysis. All mapped genes yielding fold changes greater than 1.5 on both platforms were selected for further consideration. Upon application of adjusted p-values all genes exhibiting $p<0.05$ compared to vehicle treated controls on either platforms were considered further.

2.9.2.1.GO annotations

This analysis was kindly performed by Dr R Catalano. Gene ontologies (GO) describe how gene products behave in a cellular context i.e. describe gene products in terms of their associated biological processes, cellular components and molecular functions. GO's were assigned to PROK1 regulated genes for biological processes using tools provided by the gene ontology database (www.geneontology.org).

2.9.2.2.EST tissue library

This analysis was kindly performed by Dr R Catalano. Analysis of the expressed sequence tag tissue library database for PROK1 regulated genes were analysed using WebGestalt (Web based gene set analysis toolkit) in order to examine the tissue expression of PROK1 regulated genes.

2.10. Enzyme linked immunosorbent assays (ELISA)

2.10.1. cAMP ELISA

cAMP turnover was measured in PROKR1 Ishikawa cells. Cells were seeded in 3cm dishes 24 hours prior to serum starvation. Cells were synchronised by serum withdrawal for at least 16 hours before stimulation. Thereafter, culture medium was removed and replaced with serum free medium containing 3-isobutyl-1-methyl xanthine (IBMX; Sigma) to a final concentration of 1mM for 90 minutes at 37°C. Cells were then stimulated with PROK1 for 5, 10, 15 and 20 minutes, or with forskolin for 10 minutes. Following stimulation the

medium was removed and the cells lysed in 0.1M HCl. cAMP turnover was quantified using a cAMP competitive ELISA kit (Biomol) according to the manufacturers instruction and normalised to protein concentrations of the homogenate. The assay was performed in 96 well goat anti-rabbit IgG coated microtiter plate. The cAMP standard curve was constructed by diluting the standard four times in a ratio of 1:4 in HCl (0.1M) to achieve concentrations of 200pM/ml, 50pM/ml, 12.5pM/ml, 3.13pM/ml and 0.78pM/ml which were used to construct a standard curve. The standards and samples (100µl) were loaded onto the plate. The alkaline phosphatase - cAMP conjugate and polyclonal rabbit-cAMP antibody were then added to the wells (50µl per solution). The plate was then incubated at room temperature for 2 hours on a plate shaker at approximately 500rpm. The wells were then aspirated and washed with the ELISA wash buffer (TBS-tween containing sodium azide). In order to develop the assay, the conjugate and p-Npp (p-Nitrophenyl Phosphate, Disodium Salt) substrate were added to the wells (5µl and 200µl respectively) and the plate incubated at room temperature for 1 hour. The reaction was terminated with 50µl stop solution. The absorbance was measured at 405nm using a plate reader. Average cAMP concentrations of samples were determined from the standard curve constructed by extrapolation using the Assay Zap software (Biosoft). Protein concentrations of the lysed cells were quantified using a protein assay kit (BioRad) as described in Section 2.5.1.1 and cAMP values normalised for protein concentration.

2.10.2. Prostaglandin ELISA

Secreted PGE_2 and $PGF_{2\alpha}$ from PROKR1 Ishikawa cells were measured by in house ELISA's. Cells were seeded out in 3cm dishes 24 hours prior to serum starvation. Cells were synchronised with serum withdrawal for at least 16 hours before stimulation. Thereafter cells were pre-incubated with the specific COX-2 inhibitor NS-398 (10µM) prior to stimulation with vehicle, PROK1 or PROK1 & NS-398 for 2, 4, 6, 8, 12 and 24 hours. Arachidonic acid was added at a final concentration of 3µg/ml to the media 4 hours prior to the end of the time point (i.e. For a 6 hour time point arachidonic acid was added at 2 hours). For assay of $PGF_{2\alpha}$ the media was frozen immediately while for assay

of PGE_2 an equal volume of methyloximating (MOX) solution (Methyloxyamine buffer, pH 5.6) was added to stabilise the PGE_2 prior to freezing. PGE_2 and $PGF_{2\alpha}$ secretion into the culture media was assessed using an ELISA. For this, 96-well plates (amine binding plates, Costar) were coated with donkey anti-rabbit antibody and then rabbit IgG (1mg/ml diluted in PBS with 1% carbonate buffer, pH 9.6) at 200µl/well for 16 hours at 4°C. The solution was aspirated and blocking solution (50mM glycine, 10mg/ml BSA) was added to the wells (25µl/well) for 2 hours at room temperature. Following washing, donkey anti-rabbit serum (Scottish Antibody Production Unit) was added to each well before washing and air-drying. The plates were stored at 4°C with dessicant. The link was prepared by ether extraction and purified by reverse phase chromatography using 20mg of synthetic PGE_2 or $PGF_{2\alpha}$, 320µL of dry dimethylformamide, 3µl butylchlororomate and 0.05nM biocytin. The link and antisera were diluted 1:1.5 x 10^6 in PO_4 0.5 M p H 8 and 1:50000 in ELISA buffer (150mM NaCl, 100mM Tris-HCl, 0.05% Tween 20, 50mM phenol red 1mM 2-methylisothiaolone, 1mM bromonitrodioxane, 2mM EDTA, 2mg/ml BSA to a final pH of 7.2) respectively. The prostaglandin ELISA is a competitive assay with prostaglandin within samples displacing binding of the biotin labelled prostaglandin link, therefore at the termination of the colourimetric assay the more intense the colour reaction the less prostaglandin present in the sample ie. little prostaglandin present in the sample cannot displace the binding of the biotin labelled prostaglandin therefore resulting in a more intense colour reaction. For the $PGF_{2\alpha}$ assay, the ELISA buffer contained no Tween 20. The standard (5120 pg/ml) was then serially diluted 9 times in buffer. For the PGE_2 assay the standards were diluted in ELISA buffer containing 25% MOX and Tween 20, the $PGF_{2\alpha}$ standards were diluted in ELISA buffer alone. The standards (10pg/ml up to 5120pg/ml) were used to construct the standard curve. Samples and synthetic standards (100µl) were added to the plate in duplicate followed by 50µl of the diluted link and 50µl diluted antisera to all wells except the non specific binding (NSB) and the maximum binding B_0 control wells. Link (50µl) and ELISA buffer (100µl) alone were added to the NSB wells while link (50µl); ELISA buffer (100µl) and antisera (50µl) were added to the B_o wells. The plates were incubated overnight at 4°C. The following day, the contents of the

wells were aspirated and the plates were washed 4 times, once for 30 seconds on an orbital shaker. Following the wash, 100µl/well of streptavidin-peroxidase (0.2U/ml) was added to each well on the plate. The plates were incubated for 20 minutes at 23°C on an orbital shaker. Plates were then washed and 200µl/well of the substrate (0.3g/litre urea-hydrogen peroxide, 0.1g/L tetramethylbenzene in 100mM sodium acetate, pH 6) was added for 10 minutes. The reaction was stopped with 100µl/well sulphuric acid (1M). The absorbance was measured at 450nm using a plate reader. Average PGE_2 and $PGF_{2\alpha}$ concentrations of the media samples were determined from the standard curve constructed by extrapolation using the Assay Zap software (Biosoft).

2.10.3. Estradiol and Progesterone

Estradiol was measured by commercial ELISA from Bio-Stat Diagnostics according to manufacturers instructions. Progesterone was measured according to the protocol of HM Fraser (1980). These were performed by Mr Ian Swanston.

2.10.4. LIF ELISA

Secreted LIF, from both PROKR1 Ishikawa cells and first trimester decidua was measured using an ELISA kit (R&D systems). Cells were seeded out in 3cm dishes 24 hours prior to serum starvation. Cells were synchronised with serum withdrawal for at least 16 hours before stimulation. Thereafter cells were stimulated with PROK1 or vehicle for 2, 4, 6, 8, 12 and 24 hours. Tissue was finely minced with sterile scissors and serum starved for at least 16 hours before stimulation with PROK1 or vehicle for 2, 4, 6, 8, 12 and 24 hours. Culture medium was removed and frozen at -80°C until being assayed for LIF protein using the ELISA kit as indicated above. Serial dilutions of LIF standards (3.9pg/ml up to 1000pg/ml) were made up in calibrator diluent. Standards and samples were applied to a 96 well plate coated with an antibody specific for the LIF protein. The plate was incubated for 2 hours at room temperature before washing with wash buffer supplied with the ELISA kit. A chromagen was added and the plate incubated for a further hour. Subsequently the stop solution was added in order to terminate the reaction.

The coloured product was measured on a spectrophotometer at 570nm with correction at 540nm. Samples were quantified by the construction of a standard curve using the values obtained from the standards produced by serial dilution and comparison of the samples obtained from the samples to the standard curve.

2.11. Statistics

Statistical analysis of the data in this study was carried out by ANOVA with Fishers PLSD (predicted least squares difference) applied, using Statview 5.0 (Abacus Concepts). The data is presented as mean ± SEM of at least 3 experiments.

2.12. Commonly used solutions

2.12.1. Tissue culture

Complete medium	Dulbecco's Modified Eagles Medium (DMEM) Glutamax, supplemented with: 10% (v/v) heat inactivated foetal bovine serum 100U/ml Penicillin and 100µg/ml Streptomycin 200µg/ml G418 (PROKR1 Ishikawa cells only)
Starving Medium	Dulbecco's Modified Eagles Medium (DMEM) Glutamax, supplemented with: 100U/ml Penicillin and 100µg/ml Streptomycin
Transfection medium	Dulbecco's Modified Eagles Medium (DMEM) Glutamax
Freezing medium	90% (v/v) heat inactivated foetal bovine serum 10% DMSO

2.12.2. Inositol phosphate assay

Buffer A	140 mM NaCl	8.2 g
	4 mM KCl	300 mg
	20 mM HEPES	4.8 g
	0.1% BSA (f.a. free)	1 g
	8.3 mM D-Glucose	1.5 g
	1 mM $MgCl_2$	100 mg
	1 mM $CaCl_2$	100 mg)

2.12.3. Western Immunoblotting

Lysis buffer	150mM NaCl (4.4g)
	10mM EDTA/EGTA (1.86g)
	50mM Tris pH 7.4 (25ml)
	0.6% NP40/NP40 replacement (3ml)
	10% glycerol (50ml)
	1mM Na_3VO_4
	10µg/ml pepstain
	1mM PMSF
5X Laemmli protein gel loading buffer	1M Tris pH 6.8 (6ml)
	50% Glycerol (10ml)
	10% SDS (2g)
	12.5% β-Mercaptoethanol (2.5ml)
	Trace Bromophenol blue (0.1g)
	7.5% H_2O (1.5ml)
10x Running Buffer	Tris 30.3g
	Glycine 188g
	10% SDS 100ml
	Make up to 1L with H_20

20x NuPAGE MOPS	50mM MOPS (104.6g) 50mM TRIS base (60.6g) 0.1% SDS (10g) 1mM EDTA (3g) Make up to 500ml with H_20
20x NuPAGE MES	50mM MES (97.6g) 50mM TRIS base (60.6g) 0.1% SDS (10g) 1mM EDTA (3g) Make up to 500ml with H_20
Transfer buffer	Glycine 14.4g Tris 3g 20% Methanol Make up to1L with H_20
Stripping buffer	200mM Glycine (15g) SDS (10g) Dissolve in 600ml H_2O Adjust pH to 2.5 with HCl Make up to 1L with H_2O
5x TNS - tween	NaCl 146.1g 1M TRIS HCl (100ml) 5ml Tween Make up to 1L with H_2O

2.12.4. Immunohistochemistry

NBF fixative	40% formaldehyde (100ml) Distilled water (900ml) Sodium dihydrogen phosphate monohydrate (4g) Disodium hydrogen phosphate anhydrous (6.5g)

Citrate buffer (0.1M)	Citric acid monohydrate (42.02g) Distilled water (900ml) pH to 5.5 with concentrated NaOH Make up to 2L and pH to 6
Peroxidase quenching solution	3% (v/v) Hydrogen Peroxide (30ml of 30% solution) Methanol (270ml)
Blocking solution	2ml non-immune serum 8ml PBS 0.5g BSA

Chapter 3 - Expression and localisation of PROK1 and PROKR1 in normal and pathological endometrium

3.1. Introduction

Prokineticins, PROK1 and PROK2, are pleiotropic proteins whose action appears to be tissue specific. They were initially described, and named, by Li at al (2001), for their effects in inducing contractions in the gastrointestinal tract. Subsequently, the action of PROK1 was described by LeCouter *et al* (2001) as inducing proliferation, migration and fenestrations in endothelial cells derived from endocrine glands PROK1 therefore appeared to be an angiogenic factor for endocrine gland endothelium. Examination of the expression of PROK1 in a range of human tissues revealed expression in steroidogenic tissues which undergo angiogenesis including ovary, placenta and testis (LeCouter *et al*, 2001). Delivery of PROK1 into the ovary resulted in enlargement of the ovaries with visible blood vessels and haemorrhagic areas (LeCouter *et al*, 2001). Indeed, examination of expression and localisation of PROK1 in the ovary, corpus luteum, polycystic ovaries, testis and testicular cancers suggest a role for PROK1 in angiogenesis (LeCouter *et al*, 2001, Ferrara *et al*, 2003, Samson *et al*, 2001, Kisliouk *et al*, 2005a). This is supported by studies investigating the role of PROK1 in a model of neovascularisation for age related macular degeneration and delivery of PROK1 in colorectal cancer (Tanaka *et al*, 2005, Goi *et al*, 2004). These studies demonstrated that cells overexpressing PROK1 induced neovascularisation and formation of fenestrae within these animal models.

Expression in endocrine tissues and involvement with angiogenesis led to speculation that PROK1 may be involved in endometrial angiogenesis. The endometrium is a tissue under direct influence of the steroid hormones and is one of the few tissues in the adult human which exhibit dynamic angiogenesis as part of a normal physiological process. The endometrium exhibits rapid growth, development of blood vessels, formation of glands and secretory transformation generally within a 28 - 35 day period before it is shed at menstruation and the cycle initiated again (Smith, 2001). Regeneration of the endometrium starts in the basal layer during menstruation (Rogers, 2002) and requires neovascularisation in the proliferative phase of the menstrual cycle in order to support endometrial

growth. If PROK1 were involved in endometrial neoangiogenesis, a pattern of expression in the proliferative phase of the menstrual cycle with localisation to the endothelial and smooth muscle cells would be expected. Examination of endometrial PROK1 expression, however, demonstrated an elevation in expression during the secretory phase of the menstrual cycle (Battersby *et al*, 2004a, Ngan *et al*, 2006).

Angiogenesis is also a feature of cancers; tumours are unable to grow to a size greater than around $1mm^2$ without development of a blood supply (Folkman, 1971, Gimbrove *et al*, 1972). PROK1 mRNA expression however is suggested to be down-regulated in endometrial cancer compared to normal endometrium from the mid-secretory phase of the menstrual cycle from age-matched control subjects (Ngan *et al*, 2006). This suggests that PROK1 may not play a role in neovascularistion of the endometrium; however, it may play a role in blood vessel maturation during the secretory phase of the cycle. Its potential role in early pregnancy endometrium, however, has not been examined.

This study was designed to investigate the expression and localisation of PROK1 and PROKR1 in early pregnancy endometrium (first trimester decidua) and endometrial cancer compared with normal endometrium. PROK1 or PROKR1 expressing cells were further identified by co-localisation with cluster designation markers.

3.2. Materials and Methods

3.2.1. Tissue Collection

3.2.1.1. Endometrial Tissue

Endometrial tissue collection was performed with ethical approval from Lothian Research Ethics Committee under ethics number LREC/05/S1103/32 as indicated in Section 2.2. Samples were designated according to phase of menstrual cycle as early proliferative, mid proliferative, late proliferative to ovulatory, early secretory, mid secretory and late secretory as assessed by a pathologist according to the Noyes

criterion. Circulating estradiol and Progesterone concentrations were consistent for both stated last menstrual period and histological assessment of menstrual cycle stage.

3.2.1.2. Decidua Tissue

Decidua tissue collection was performed with ethical approval from Lothian Research Ethics Committee under ethics number LREC/05/S1103/32 as indicated in Section 2.2. Gestation of pregnancy was confirmed by ultrasound scan prior to elective first trimester surgical termination of pregnancy

3.2.1.3. Endometrial Cancer Tissue

Endometrial cancer tissue collection was performed with ethical approval from Lothian Research Ethics Committee under ethics number LREC/1999/6/4 as indicated in Section 2.2. Endometrial cancer tissue was obtained from women undergoing surgery for removal of endometrial cancer. Cancer biopsies were assessed by a pathologist and designated as grade 1: well differentiated, grade 2: moderately differentiated or grade 3: poorly differentiated.

Written informed consent was obtained from all subjects prior to tissue collection. Shortly after collection, tissue was placed in RNAlater and stored at -70°C (for RNA extraction), fixed in neutral buffered formalin and wax embedded (for immunohistochemical analysis).

3.2.2. Polymerase chain reaction

3.2.2.1. RNA extraction

RNA was extracted using TRI-reagent and the phenol-chloroform method of RNA extraction as described in section 2.5. Briefly, TRI-reagent was added to tissue in 2.0ml Eppendorf tubes containing a 5mm stainless steel bead. Tissue was then homogenized using a tissue lyser (Qiagen) subjecting the samples to shaking at 25Hz for 6 minutes. Homogenised tissue samples were then cleared of cellular debris by centrifugation at

15000 RPM for 10 minutes at 4°C. Lysed tissue samples were then loaded into heavy gel phase lock tubes with the addition of bromo-chloro propane. Tubes were shaken vigorously to ensure adequate mixing of the two solutions. Samples were then processed as described in section 2.5.

3.2.2.2. RT-PCR

uNK cell RNA was quantified and prepared at a concentration of 1μg in a volume of 10μl in order to make cDNA as described in section 2.5. In order to amplify PROK1 cDNA, PCR was performed using specific PROK1 primers: -

Forward - 5′ GTG CCA CCC GGG CAG 3′

Reverse -5′ AGC AAG GAC AGG TGT GGT GC 3′

A 1% Tris-Acetate-EDTA (TAE) agarose gel containing ethidium bromide was then prepared and samples run for approximately 1 hour 30 minutes before visualisation using a UV transilluminator.

3.2.2.3.Taqman quantitative PCR analysis

Expression of RNA within tissue was examined by Taqman quantitative PCR analysis as described in section 2.5. Briefly, cDNA was prepared in a random hexamer primed reaction using 200ng RNA per reaction. Quantitative PCR analysis was performed on the cDNA using specific primer probe combinations to detect PROK1 or PROKR1 (sequences given in Table 5). These were designed in house and were custom synthesised. The probe was given a FAM fluorescent label with a TAMRA quencher. Gene expression was normalized by including Vic labelled primer-probe mix to detect the 18s ribosomal subunit (sequences given in Table 5), as a loading control for the amount of cDNA added in each sample.

Reaction mixes were loaded in duplicate onto a 96-well MicroAmp fast optical reaction plate for analysis on an ABI7900 HT Fast Real-Time PCR machine. Data were analysed and processed using sequence detector version 1.6.3. Expression level of the gene of interest was expressed relative

to an internal positive RNA standard included in all reactions. The data were analysed using the comparative C_T method for relative quantification.

3.2.3. Immunohistochemistry

3.2.3.1.DAB immunohistochemistry

Tissue sections (5μM) were dewaxed in xylene and sequentially rehydrated in decreasing concentrations of ethanol (100%, 95%, 80%, 75%) and finally placed in water when rehydration was complete. Antigen retrieval was performed in order to reveal the epitope sites of the proteins and make them available for the antibodies to bind. This was achieved by placing the rehydrated tissue sections in a pressure cooker followed by boiling for five minutes in 0.1% citrate buffer (pH 6.0). The sections were allowed to rest and cool for 20 minutes before adding excess cold water. Endogenous peroxidase activity was quenched with 3% (vol/vol) H_2O_2 in methanol at room temperature. Blocking serum (5% normal swine serum in Tris-buffered saline with 0.05% BSA) was applied for one hour in order to block any non-specific binding. Subsequently PROK1 (1:1000) or PROKR1 (1:250) antibody was applied in the serum block solution and incubated overnight at 4°C. The tissue sections were then washed sequentially in tris neutral saline (TNS)-tween and TNS to remove any excess antibody before application of goat anti-rabbit biotinylated antibody. The sections were washed in TNS and exposed to the ABC reagent (Dako) for 30 minutes. The sections were again washed in TNS before exposure to the chromagen 3,3"-diaminobenzidine, the colour development was checked under a microscope and the reaction terminated by placing the slides in water. The slides were counterstained with haematoxylin and dehydrated in increasing concentrations of ethanol (75%, 80%, 95%, 100%). Sections were then mounted in pertex. Negative controls were incubated with isotype matched IgG in place of primary antibody.

3.2.3.2. Immunofluorescent histochemistry and confocal microscopy

Tissue sections (5μM) were dewaxed in xylene, rehydrated and subjected to antigen retrieval followed by endogenous peroxidase quench with 3%

(vol/vol) H_2O_2 in methanol at room temperature as described in Section 3.2.3.1. Sections were then blocked in 5% normal goat serum block (5% normal goat serum in phosphate-buffered saline with 0.05% BSA) followed by incubation overnight at 4°C with CD56 (1:250), CD68 (1:30) or CD31 (1:30). Sections were washed in PBS-tween followed by PBS, incubated for one hour with goat anti-mouse biotinylated antibody (1:500) followed by incubation with Streptavidin 488 (green) or 546 (red) in PBS (1:200). Sections were subsequently re-blocked with 5% goat serum block followed by incubation with PROK1 (1:1500) or PROKR1 (1:500) overnight at 4°C. Sections were washed in PBS-tween followed by PBS, incubated for one hour with goat anti-rabbit peroxidase antibody (1:200) followed by incubation with TSA plus Fluorescein (green) or Cyanine (red) at 1:50 in substrate for 10 minutes. Sections were then incubated with the nuclear counterstain ToPro (1:2000) for 2 minutes, mounted in Permafluor and coverslipped. Fluorescent images were visualised using a laser-scanning confocal microscope (LSM 510, Carl Zeiss, Jena, Germany) with a 40 x 1.4 aperture oil immersion lens. Negative controls were incubated with isotype matched IgG in place of primary antibody.

3.2.4. Statistics

Statistical analysis of the data in this study was carried out by ANOVA with Fishers PLSD (predicted least squares difference) applied, using Statview 5.0 (Abacus Concepts). The data is presented as mean ± SEM of at least 3 experiments.

3.3. Results

3.3.1. Expression and localisation of PROK1 in normal cycling endometrium and early pregnancy decidua.

3.3.1.1. Expression of PROK1 throughout the menstrual cycle

Endometrial samples taken across the menstrual cycle were analysed for expression of PROK1 mRNA by quantitative RT-PCR analysis. PROK1 mRNA expression in secretory phase endometrial samples was

significantly elevated when compared with proliferative phase endometrial samples ($p<0.05$, Figure 3.1) with an apparent peak in expression during the mid-secretory phase of the menstrual cycle.

3.3.1.2.Expression of PROK1 in first trimester decidua

First trimester decidua samples taken from weeks 7 - 12 of pregnancy were analysed for expression of PROK1. All samples examined expressed PROK1 mRNA however the expression of PROK1 was variable (Figure 3.2 A). Sample numbers from each phase of gestation were insufficient to make comparisons between gestational ages and as the date allocated by the scan may vary by 3 days either side it is difficult to be completely certain that the gestational date assigned is correct. However, when PROK1 mRNA expression data from all first trimester decidua were pooled and compared with PROK1 mRNA expression in the normal cycling endometrium (from proliferative or secretory phase) it was revealed that PROK1 expression was significantly elevated in the pregnant compared with the non-pregnant endometrium ($p<0.001$, Figure 3.2 B).

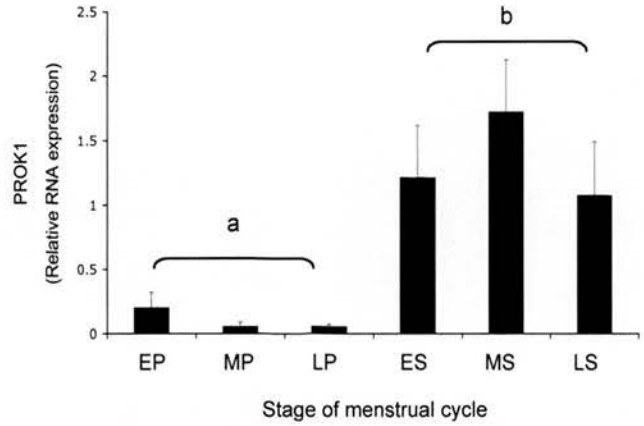


Figure 3.1. Expression of PROK1 across the menstrual cycle. Samples of endometrial tissue taken from the early proliferative (EP, n=7) mid proliferative (MP, n=9) late proliferative (n=7), early secretory (ES, n=11), mid secretory (MS, n=12) and late secretory (LS, n=5) were examined for the expression of PROK1 by Taqman quantitative PCR analysis. PROK1 expression was normalized for loading against expression of 18s and relative to a single endometrial control. Data are presented as mean ± SEM. (a is significantly different from b $p<0.05$). Stage of the menstrual cycle was assessed in tissues by a pathologist according to the Noyes criterion.

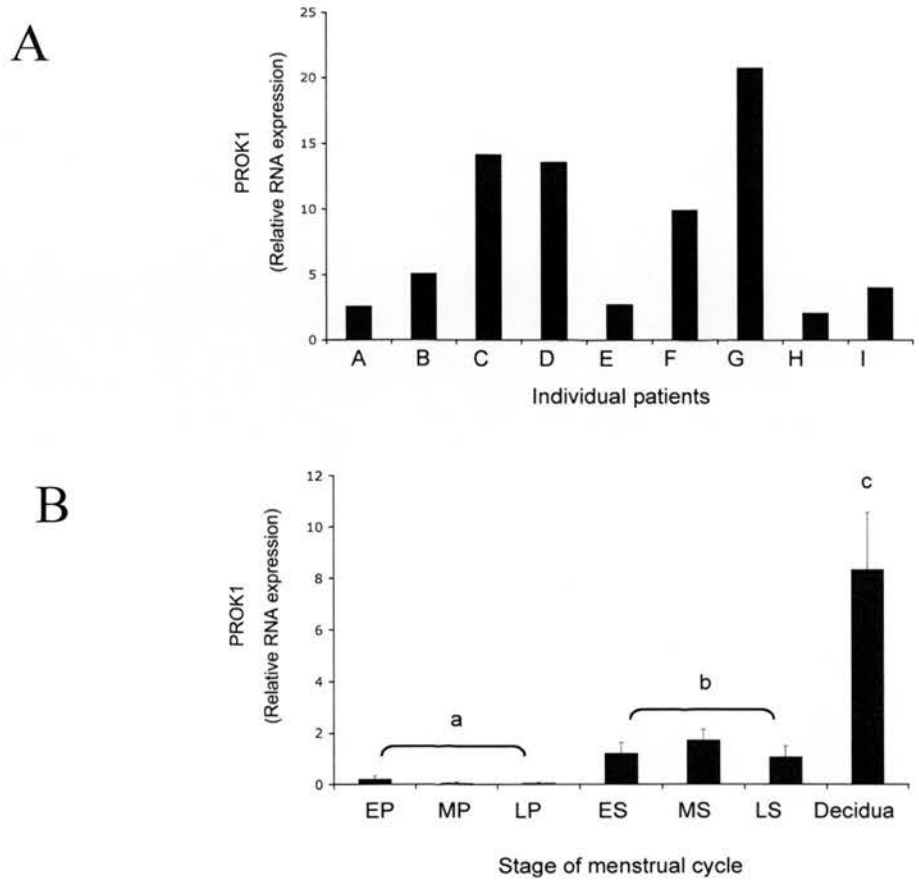


Figure 3.2. Expression of PROK1 in first trimester decidua and comparison with PROK1 expression across the menstrual cycle. A, samples of first trimester decidua tissue taken between weeks 7 – 12 of gestation were examined for expression of PROK1 by Taqman quantitative PCR analysis. PROK1 expression was normalized for loading against expression of 18s and relative to an internal endometrial control. B, samples of endometrial tissue taken from the early proliferative (EP, n=7) mid proliferative (MP, n=9) late proliferative (LP, n=7), early secretory (ES, n=11), mid secretory (MS, n=12), late secretory (LS, n=5) and first trimester decidua (n=9) were examined for the expression of PROK1 by Taqman quantitative PCR analysis. PROK1 expression was normalized for loading against expression of 18s and relative to a single endometrial control. Data are presented as mean ± SEM. (a is significantly different from b $p<0.05$, c is different from a and b $p<0.001$). Stage of the menstrual cycle was assessed in tissues by a pathologist according to the Noyes criterion.

3.3.1.3. Locaisation of PROK1 in normal endometrium and first trimester decidua

Immunohistochemical localisation of PROK1 in the endometrium has been described previously (Battersby *et al*, 2004a); therefore only one sample of secretory phase endometrium is shown. In agreement with previous reports

staining for PROK1 protein is observed in the glandular epithelium and stromal cells (Figure 3.3, A and B). However, only very weak staining of endothelial cells was observed (Figure 3.3, B indicated by arrowhead). In the first trimester decidua tissue, staining and localisation of PROK1 protein was similar in all samples examined (n=7), therefore only one sample is presented as an illustration. In first trimester decidua the PROK1 localisation is similar to the non-pregnant endometrium with staining detected in the glandular epithelium and decidualised stromal cells. However, extensive staining of the endothelial cells of the microvasculature is also present in the pregnant endometrium (Figure 3.3 C and D). Examination of one first trimester decidua sample contaminated with trophoblast revealed localisation of PROK1 protein to the cytotrophoblast and syncytiotrophoblast cells of the first trimester (Figure 3.3 E and F). The negative controls were incubated with isotype matched IgG in place of primary antibody and displayed no immunoreactivity (Figure 3.3 G and H).

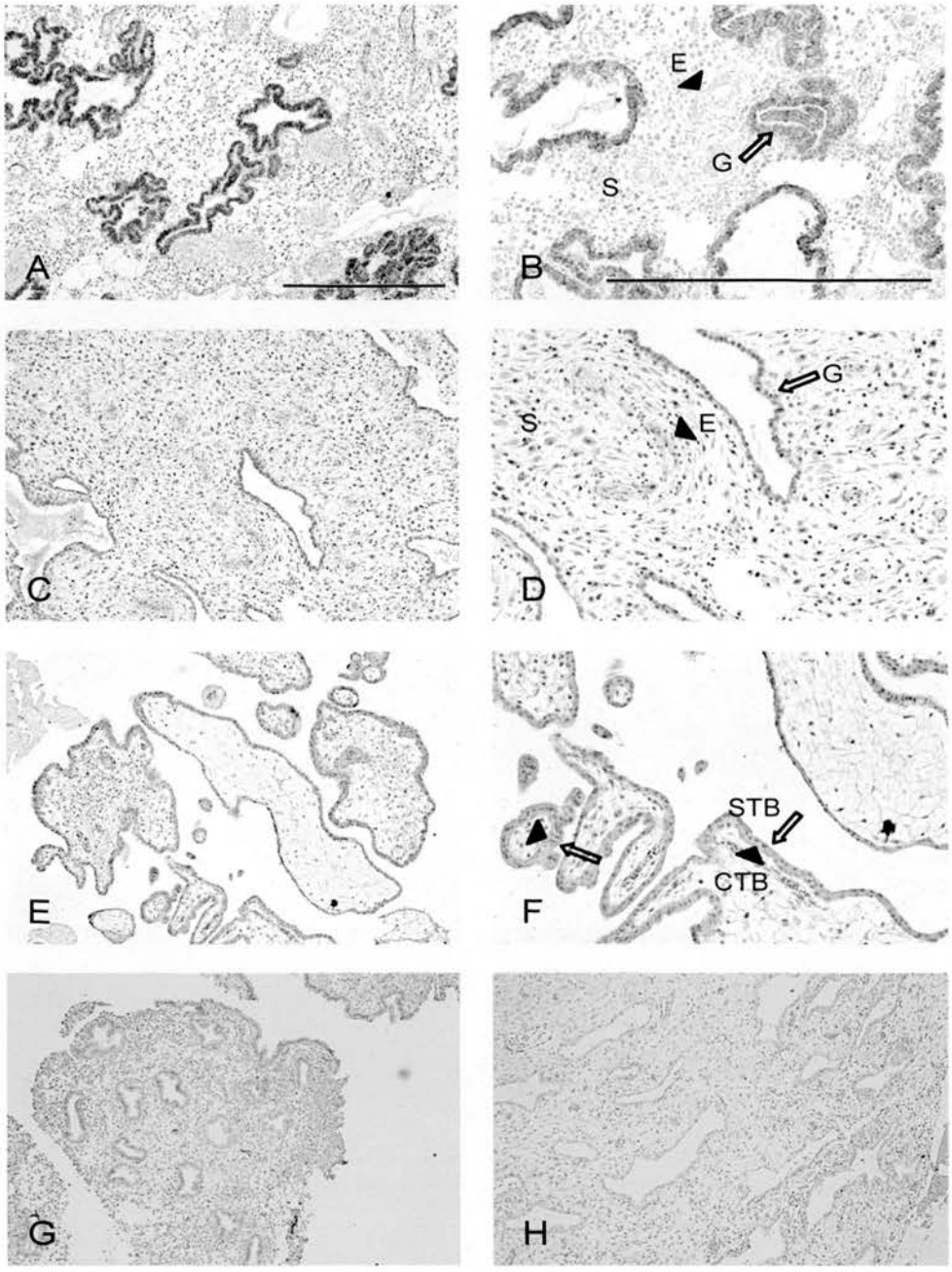


Figure 3.3. Localisation of PROK1 in mid-secretory endometrium (A and B) and first trimester decidua (C, D, E and F). PROK1 (A and B) localises to the glandular epithelial cells (G, indicated by arrow) stromal cells (S) and weakly to the endothelial cells (E) of the endometrium (A is x10 magnification, B is x20 magnification, scale bar indicates 50μM in each image). PROK1 expression in first trimester decidua (C and D) localises to the glandular epithelial cells (G) stromal cells (S) and endothelial cells (E) (C is x10 magnification, B is x20 magnification). One first trimester decidua sample contaminated with trophoblast revealed PROK1 expression within the trophoblast layers (E and F) localising to the syncytiotrophoblast (STB) and cytotrophoblast (CTB) layers of the invading trophoblast (E is x10 magnification, F is x20 magnification). Endometrium (G) and first trimester decidua (H) negative controls were incubated with IgG in place of the primary antibody, with no immunoreactivity detected.

3.3.2. Expression and localisation of PROKR1 in normal cycling endometrium and early pregnancy decidua

3.3.2.1. Expression of PROKR1 throughout the menstrual cycle

Endometrial samples taken across the menstrual cycle were analysed for expression of PROKR1 by quantitative RT-PCR. PROKR1 mRNA expression did not alter significantly across the menstrual cycle (Figure 3.4).

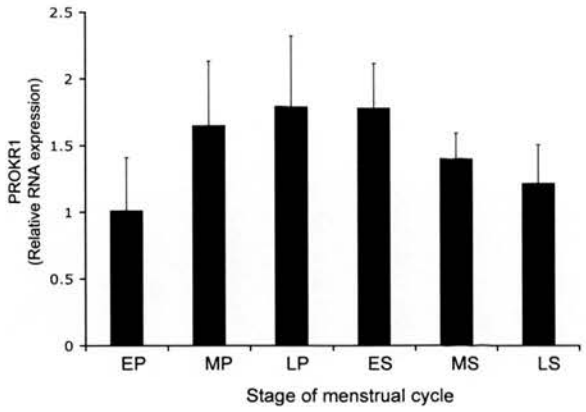


Figure 3.4. Expression of PROKR1 across the menstrual cycle. Samples of endometrial tissue taken from the early proliferative (EP, n=7) mid proliferative (MP, n=9) late proliferative (n=7), early secretory (ES, n=11), mid secretory (MS, n=12) and late secretory (LS, n=5) were examined for the expression of PROKR1 by Taqman quantitative PCR analysis. PROKR1 expression was normalized for loading against expression of 18s and relative to a single endometrial control. Data are presented as mean ± SEM. Stage of the menstrual cycle was assessed in tissues by a pathologist according to the Noyes criterion.

3.3.2.2. Expression of PROKR1 in first trimester decidua

First trimester decidua samples were analysed for expression of PROKR1. All samples examined expressed PROKR1 mRNA (Figure 3.5 A). Pooling of these data and comparison to PROKR1 mRNA expression in the normal cycling endometrium (from proliferative or secretory phase) revealed that PROKR1 expression was significantly elevated in the pregnant compared with the non-pregnant endometrium (Figure 3.5 B, $p<0.001$)

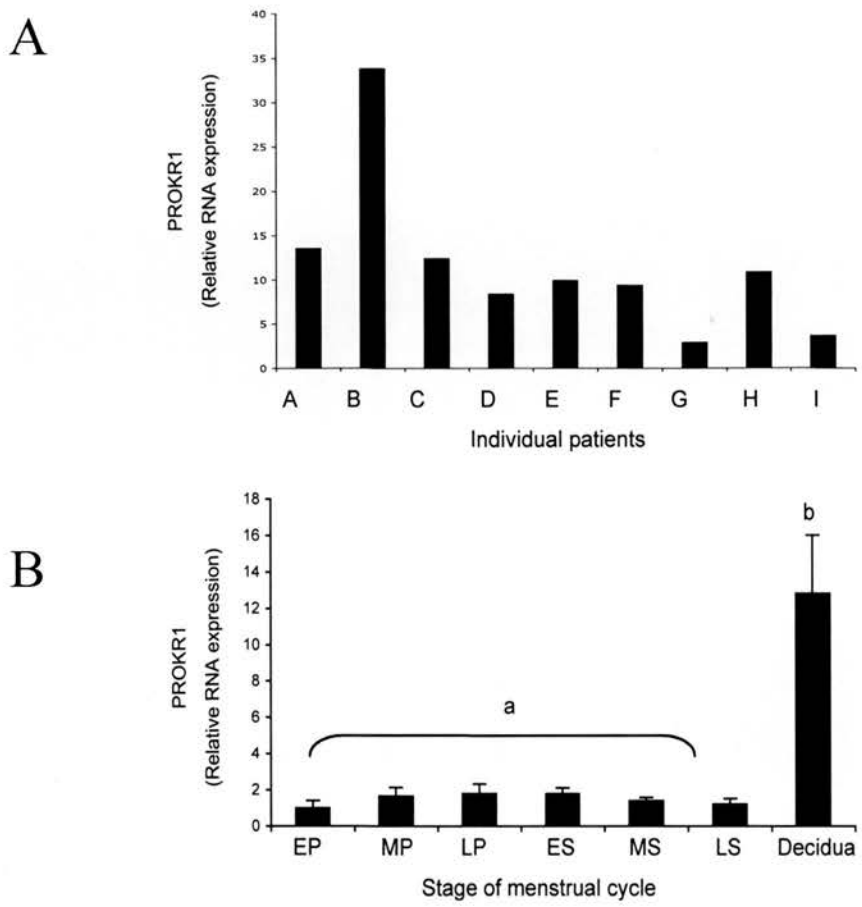


Figure 3.5. Expression of PROKR1 in first trimester decidua and comparison with PROK1 expression across the menstrual cycle. A, samples of first trimester decidua tissue taken between weeks 7 – 12 of gestation were examined for expression of PROKR1 by Taqman quantitative PCR analysis. PROKR1 expression was normalized for loading against expression of 18s and relative to an internal endometrial control. B, samples of endometrial tissue taken from the early proliferative (EP, n=7) mid proliferative (MP, n=9) late proliferative (n=7), early secretory (ES, n=11), mid secretory (MS, n=12), late secretory (LS, n=5) and first trimester decidua (n=9) were examined for the expression of PROKR1 by Taqman quantitative PCR analysis. PROKR1 expression was normalized for loading against expression of 18s and relative to a single endometrial control. Data are presented as mean ± SEM. (a is significantly different from b $p<0.001$). Stage of the menstrual cycle was assessed in tissues by a pathologist according to the Noyes criterion.

3.3.2.3. Localisation of PROKR1 in normal endometrium and first trimester decidua

PROKR1 expression in the human endometrium has previously been localised by in situ hybridisation. In this study expression of endometrial PROKR1 protein was investigated by immunohistochemistry in endometrium samples collected across the menstrual cycle. Examination of PROKR1 mRNA revealed no variation across the menstrual cycle while immunohistochemical analysis appeared to demonstrate greater staining in samples of secretory phase endometrium compared to proliferative endometrium, however a large degree of inter-sample variation was evident. PROKR1 localises to the glandular epithelial cells of proliferative endometrium with no immunoreactivity detected in stromal or endothelial cells (Figure 3.6 A and B). In early secretory endometrium PROKR1 again localises to the glandular epithelial cells, however, immunoreactivity becomes apparent in some stromal cells with limited immunoreactivity also noted in the endothelial cells (Figure 3.6 C and D). In mid-secretory endometrium PROKR1 localises to the glandular epithelial cells and endothelial cells of the microvasculature along with extensive stromal cell staining (Figure 3.6 E and F). In first trimester decidua (n=7) PROKR1 localisation is similar to that revealed in mid-secretory endometrium, with localisation to the glandular epithelium, decidualised stromal cells and endothelial cells (Figure 3.7 A and B). Examination of the first trimester decidua sample contaminated with trophoblast also reveals expression of PROKR1 in the cytotrophoblast and syncytiotrophoblast cells of the first trimester (Figure 3.7 C and D). Negative controls were incubated with the primary antibody pre-absorbed with neutralising peptide or with isotype matched IgG in place of the primary antibody. These exhibited no immunoreactivity (Figure 3.6 inset F, Figure 3.7 E and F).

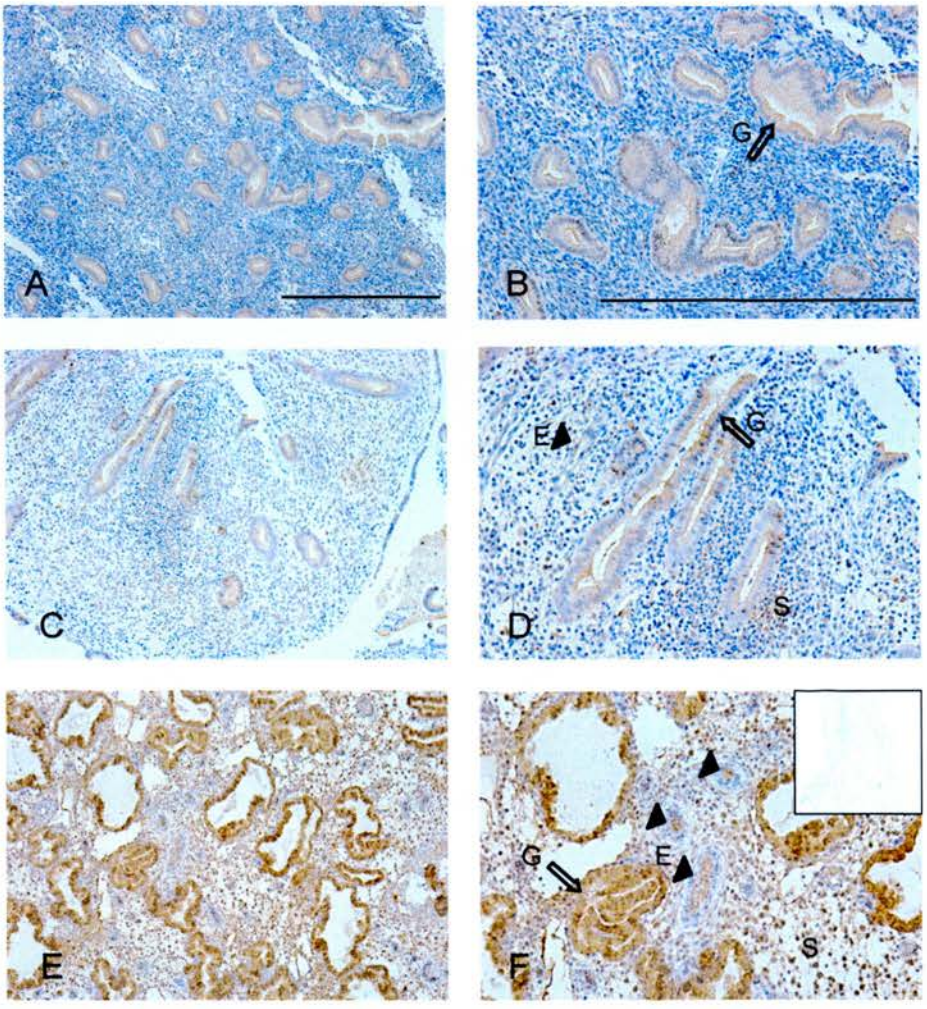


Figure 3.6. Localisation of PROKR1 in human endometrium in mid-proliferative (A and B), early secretory (C and D) and mid secretory (E and F) phases of the menstrual cycle. PROKR1 (A and B) was localised to the glandular epithelial cells of the endometrium (G) in the mid-proliferative phase of the menstrual cycle (A is x10 magnification, B is x20 magnification, scale bars indicate 50μM in each image). PROKR1 (C and D) was localised to the glandular epithelial cells (G) with appearance in endothelial cells (E) and some stromal cells (S) in the early secretory phase of the cycle (C is x10 magnification, D is x20 magnification). PROKR1 was localised to the glandular epithelium (G) with extensive stromal cell (S) and endothelial cell (E) localisation in the mid-secretory phase of the menstrual cycle (E is x10 magnification, F is x20 magnification). Inset F is negative control in which sections were treated with PROKR1 antibody pre-absorbed with neutralising peptide (Lifespan Biosciences, at a x10 excess concentration to antibody); no immunoreactivity was detected. Stage of the menstrual cycle was assessed in tissues by a pathologist according to the Noyes criterion.

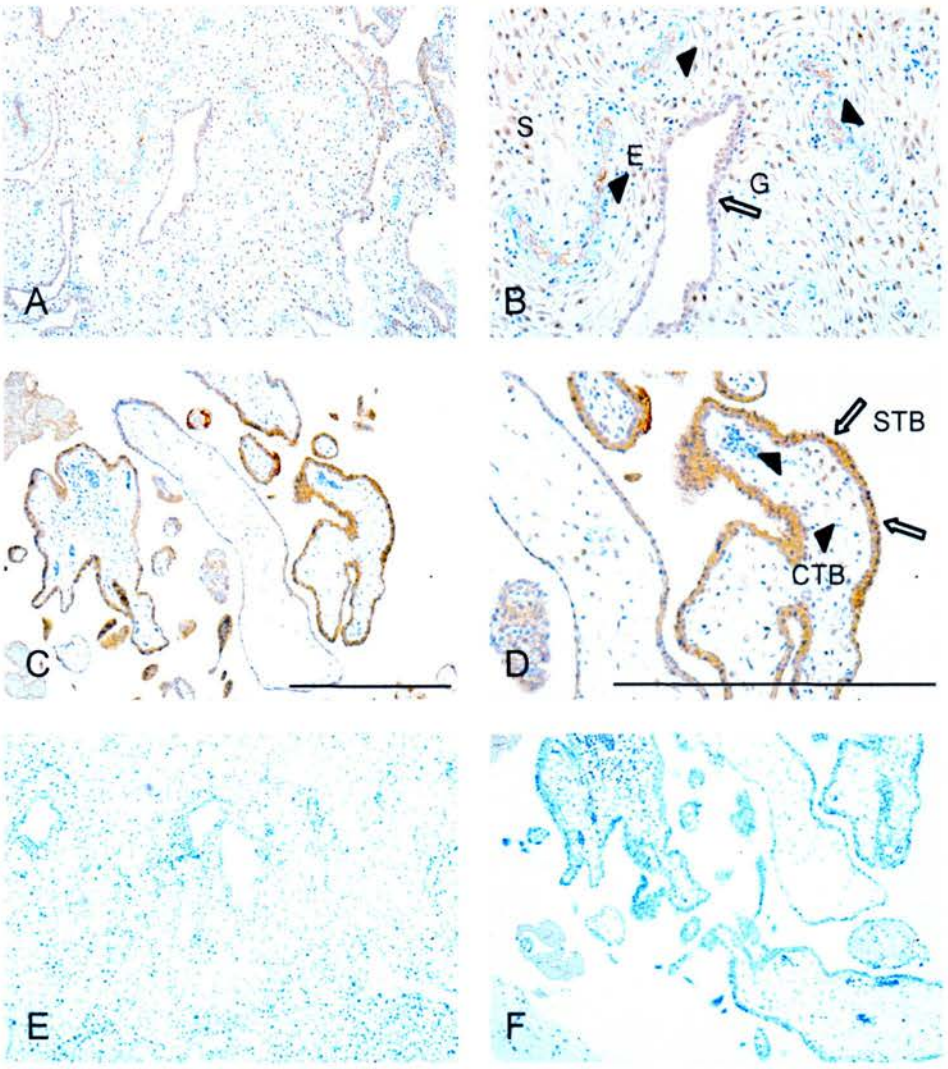


Figure 3.7. Localisation of PROKR1 in first trimester decidua. PROKR1 (A and B) localises to the glandular epithelial cells (G), stromal cells (S) and endothelial cells (E) of first trimester decidua tissue (A is x10 magnification, B is x20 magnification). One first trimester decidua sample contaminated with trophoblast revealed PROKR1 expression within the trophoblast layers (C and D) localising to the syncytiotrophoblast (STB) and cytotrophoblast (CTB) layers of the invading trophoblast (C is x10 magnification, D is x20 magnification, scale bars indicate 50μM in each image). First trimester decidua (E) and trophoblast (F) negative controls were incubated with IgG in place of the primary antibody, with no immunoreactivity detected.

3.3.3. Identification of PROK1/PROKR1 expressing endometrial cells

3.3.3.1. Expression of PROK1 and PROKR1 in immune cells within the endometrium

During the peri-implantation phase of the menstrual cycle and during early pregnancy, when PROK1 expression is elevated, immune cells infiltrate the endometrium in great numbers. As PROK1 and PROKR1 expression is elevated in first trimester decidua with localization detected in the stroma where the immune cells reside it was decided to investigate whether populations of stromal cells that express PROK1 and PROKR1 were immune cells. Immunofluorescent co-localisation conducted on decidua using the uterine natural killer (uNK) cell marker CD56 (red channel) revealed clusters of PROK1 expressing stromal cells (green channel) to be uNK cells (yellow channel, Figure 3.8 A). Further confirmation of PROK1 expression by uNK cells was obtained by RT-PCR analysis conducted on RNA extracted from uNK cells isolated from 3 individual decidua samples (kindly provided by Dr N Kane, Figure 3.8 B). PROKR1 however did not localise to uNK cells and PCR analysis confirmed that PROKR1 is not expressed in uNK cells (data not shown). Further investigation of the stromal cell population revealed PROK1 (red channel) and PROKR1 (red channel) expression to be localised to the CD68 (green channel) expressing macrophages (yellow channel) present within the decidual stroma by immunofluorescent co-localisation (Figure 3.9 and 3.10 respectively). Tissue sections were incubated with isotype matched IgG in place of the primary antibody and displayed no immunoreactivity.

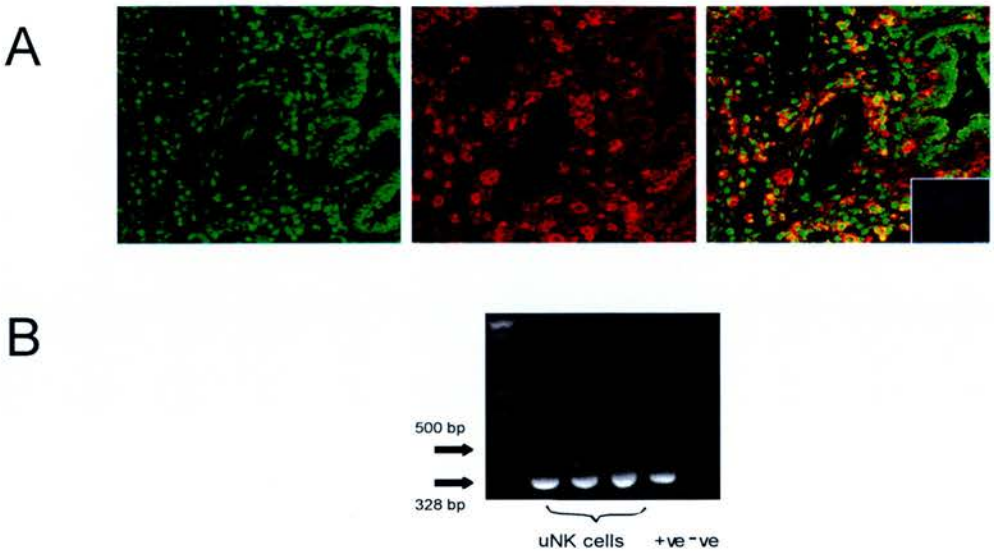


Figure 3.8. Expression of PROK1 by uNK cells. A, PROK1 (green) and uNK cell marker (CD56, red) were co-localised (yellow, merge) to stromal cells surrounding blood vessels in decidua. Inset, negative control was incubated with isotype matched IgG in place of first and second antibody and demonstrated no immunoreactivity. B, RT-PCR analysis conducted on uNK cells isolated from 3 different decidua using specific primers for PROK1. Positive control is endometrium; negative control is endometrium with no Taq polymerase enzyme in the RT-PCR reaction.

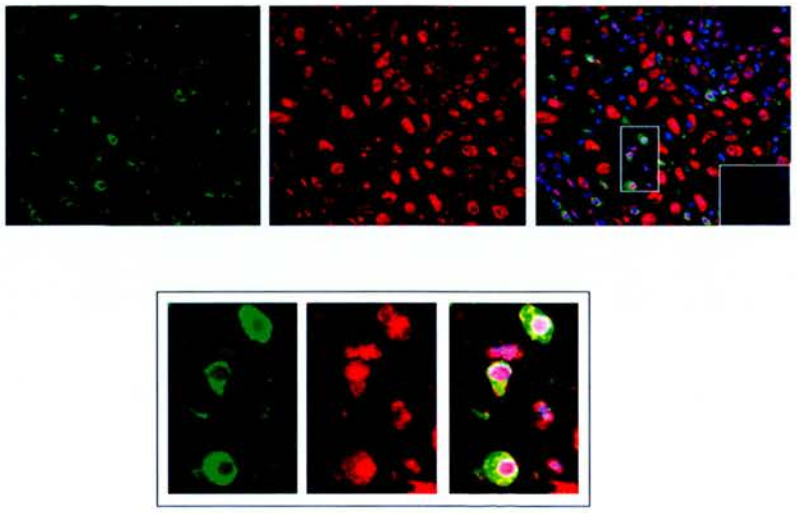


Figure 3.9. Localisation of PROK1 to macrophages within decidua. PROK1 (red) and macrophage marker (CD68, green) were co-localised (yellow, merge) to isolated cells within the decidual stroma. The lower picture presents a magnified view of 3 macrophages within the isolated white box. Inset, negative control was incubated with isotype matched IgG in place of first and second antibodies with no immunoreactivity detected.

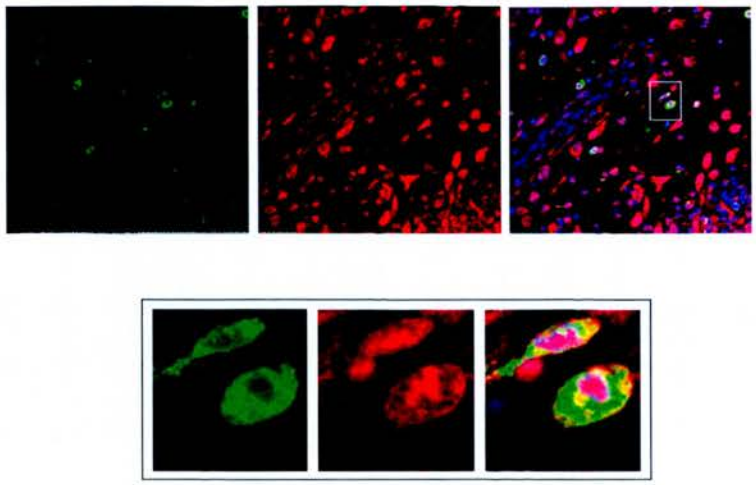


Figure 3.10. Localisation of PROKR1 to macrophages within decidua. PROKR1 (red) and macrophage marker (CD68, green) were co-localised (yellow, merge) to isolated cells within the decidual stroma. The lower picture presents a magnified view of 2 macrophages within the isolated white box. Inset, negative control was incubated with isotype matched IgG in place of first and second antibodies with no immunoreactivity detected.

3.3.3.2. Expression of PROKR1 in endometrial endothelial cells

DAB immunohistochemistry staining localised PROKR1 to the endothelial cells of the endometrium. In order to confirm the expression of PROKR1 in endothelial cells, PROKR1 (green channel) and the endothelial cell marker CD31 (red channel) were co-localised by immunofluorescent histochemistry (yellow channel, Figure 3.11). This confirmed the endothelial cell expression of PROKR1.

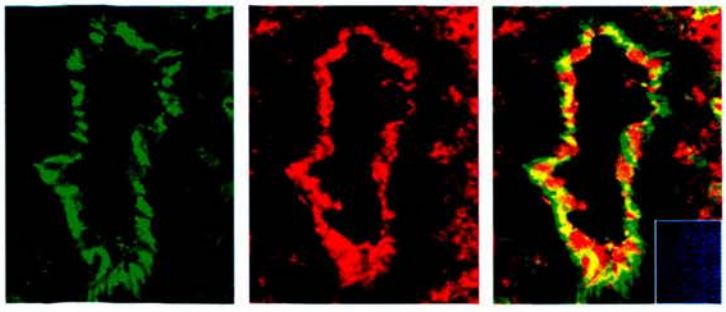


Figure 3.11. Localisation of PROKR1 to endothelial cells of the endometrium. PROKR1 (green) and endothelial cell marker (CD31, red) co-localise (yellow, merge) to the endothelial cells of the endometrium. Inset, isotype matched IgG in place of first and second antibodies negative control.

3.3.4. Expression and localisation of PROK1 in endometrial cancer

3.3.4.1. Expression of PROK1 in well, moderately and poorly differentiated endometrial cancer

Examination of PROK1 mRNA expression by quantitative PCR in endometrial cancer samples, representing well, moderately and poorly differentiated carcinoma revealed no difference in expression between these different stages (Figure 3.12 A). The levels of PROK1 expression in endometrial cancer are higher than those for PROK1 in the proliferative phase of the menstrual cycle but lower than the levels of PROK1 expression in the secretory phase of the cycle (Figure 3.12 B).

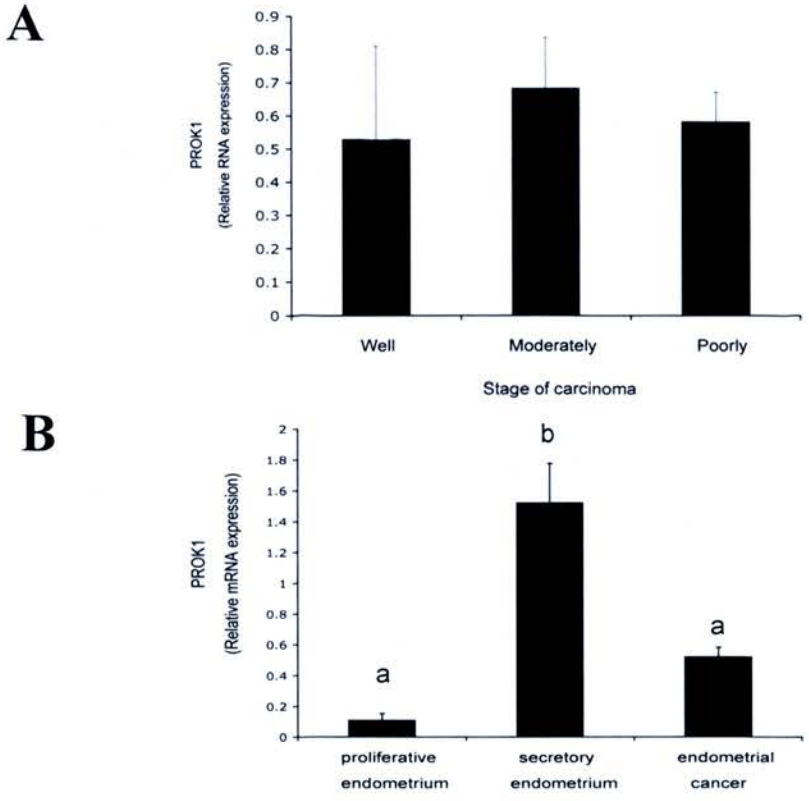


Figure 3.12. Expression of PROK1 in endometrial cancer and normal cycling endometrium. A. Samples of endometrial cancer tissue from well differentiated (n=4), moderately differentiated (n=8) and poorly differentiated (n=7) endometrial carcinomas were examined for the expression of PROK1 by Taqman quantitative PCR analysis. B. Samples of endometrium from the proliferative and secretory phases of the endometrium in addition to samples of endometrial carcinoma were examined for PROK1 expression by Taqman quantitative PCR analysis. PROK1 expression was normalized for loading against expression of 18s and relative to a single endometrial control. PROK1

expression is significantly elevated in secretory phase endometrium compared with proliferative endometrium and endometrial cancer. Data are presented as mean ± SEM (a is significantly different from b $p<0.05$).

3.3.4.2. Localisation of PROK1 in well, moderately and poorly differentiated endometrial cancer

Immunohistochemical analysis of endometrial cancer revealed some PROK1 protein expression in glandular epithelial cells present in well-differentiated carcinoma (Figure 3.13 A and B), however PROK1 protein expression is almost completely absent in moderately (Figure 3.13 C and D) and poorly (Figure 3.13 E) differentiated carcinoma samples. Negative controls were incubated with isotype matched IgG in place of primary antibody and displayed no immunoreactivity (Figure 3.13 F, G and H representing negative controls for well, moderately and poorly differentiated carcinoma respectively).

3.3.5. Expression and localisation of PROKR1 in endometrial cancer

3.3.5.1. Expression of PROKR1 in well, moderately and poorly differentiated endometrial cancer

Examination of PROKR1 mRNA expression by quantitative PCR analysis in endometrial cancer samples, representing well, moderately and poorly differentiated carcinoma revealed no difference in expression between these different stages (Figure 3.14 A). The levels of PROKR1 in endometrial cancer are similar to those for PROKR1 throughout the menstrual cycle (Figure 3.14 B).

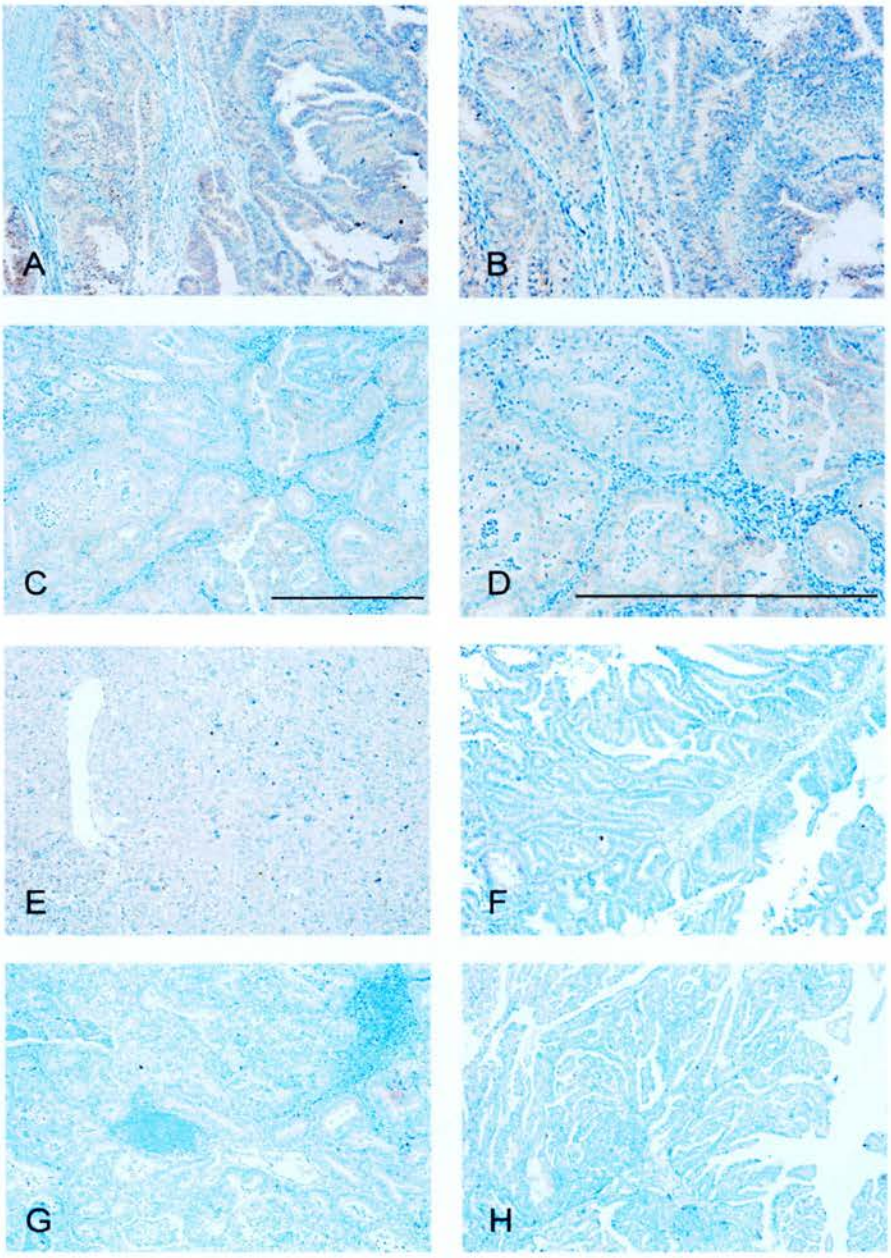


Figure 3.13. Localisation of PROK1 in endometrial cancer from well (A and B), moderately (C and D) and poorly (E) differentiated endometrial cancer samples. PROK1 appears to localise weakly to gland-like structures in well (A x10 magnification, and B x20 magnification) and moderately (C x10 magnification and D x20 magnification, scale bars indicate 50μM in each image) differentiated endometrial cancer samples). No PROK1 immunoreactivity was present in poorly differentiated (E x10 magnification) cancer samples. Negative controls from well (F), moderately (G) and poorly (H) differentiated cancer samples were incubated with IgG in place of primary antibody with no immunoreactivity detected.

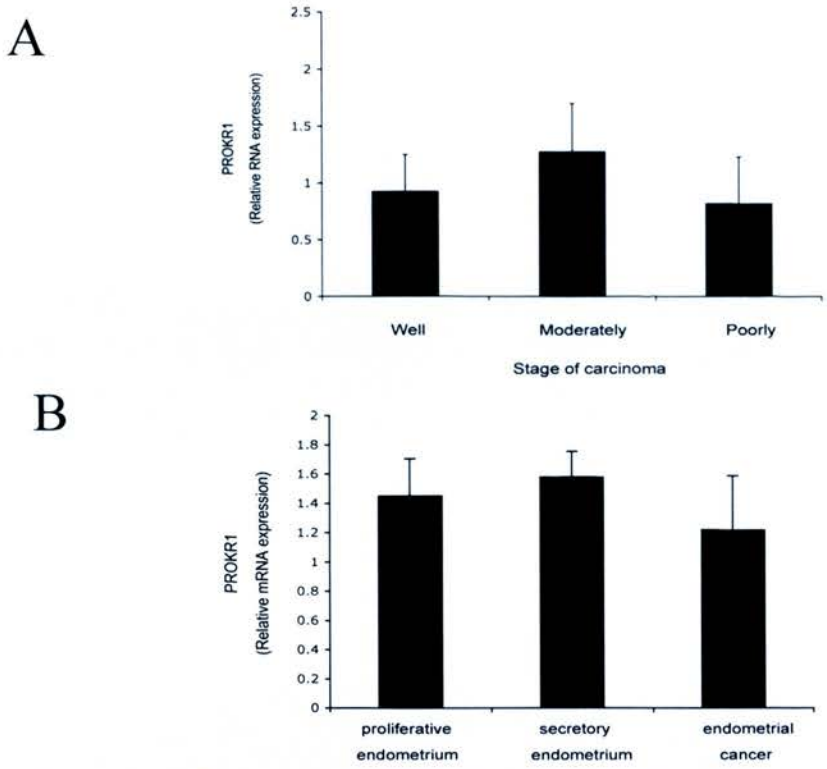


Figure 3.14. Expression of PROKR1 in endometrial cancer and normal cycling endometrium. Samples of endometrial cancer tissue from well differentiated (n=4), moderately differentiated (n=8) and poorly differentiated (n=7) endometrial carcinomas were examined for the expression of PROKR1 by Taqman quantitative PCR analysis. B. Samples of endometrium from the proliferative and secretory phases of the endometrium in addition to samples of endometrial carcinoma were examined for PROKR1 expression by Taqman quantitative PCR analysis. PROKR1 expression was normalized for loading against expression of 18s and relative to a single endometrial control. Data are presented as mean ± SEM.

3.3.5.2. Localisation of PROKR1 in well, moderately and poorly differentiated endometrial cancer

Immunohistochemical analysis of endometrial cancer revealed PROKR1 protein expression to be almost absent in well differentiated carcinoma samples (Figure 3.15 A and B), however some immunoreactivity was revealed in moderately differentiated samples, possibly in gland-like structures (Figure 3.15 C and D). PROKR1 protein expression is almost completely absent in poorly differentiated carcinomas (Figure 3.15 E and F). Negative controls were incubated with isotype matched IgG in place of primary antibody and displayed no immunoreactivity (Figure 3.15 G and H).

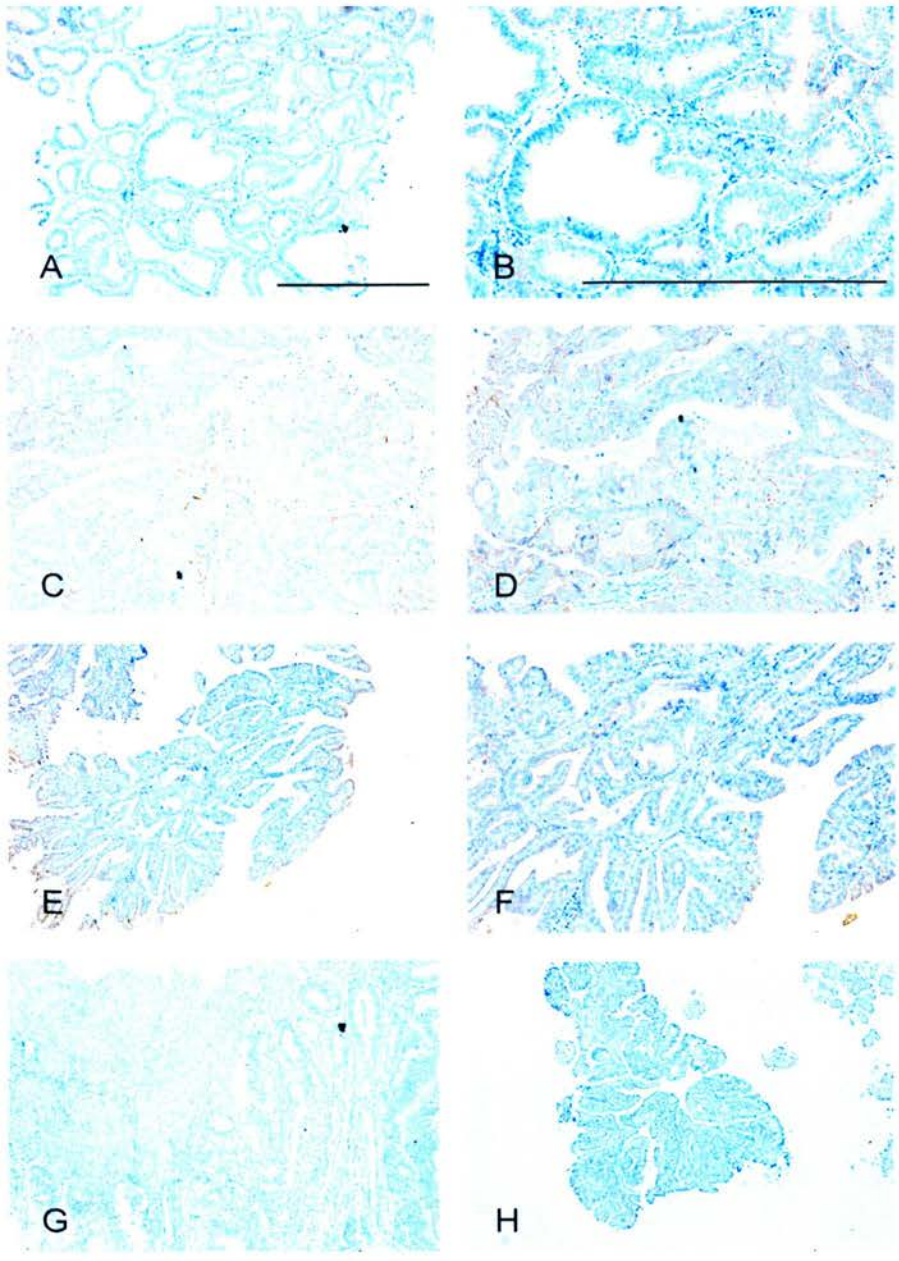


Figure 3.15. Localisation of PROKR1 in endometrial cancer from well (A x10 magnification, B x20 magnification, scale bars indicate 50μM in each image), moderately (C and D) and poorly (E and F) differentiated endometrial cancer samples. PROKR1 appears to localise weakly to gland-like structures in moderately (C x10 magnification and D x20 magnification) differentiated endometrial cancer samples. Well and poorly differentiated cancer samples display little immunoreactivity. Negative controls from moderately (G) and poorly (H) differentiated cancer samples were incubated with IgG in place of primary antibody with no immunoreactivity detected.

3.4. Discussion

This study characterises the expression and localisation of PROK1 and PROKR1 in the pregnant and non-pregnant endometrium and also endometrial carcinoma. A population of PROK1 and/or PROKR1 expressing stromal cells have been further identified as bone marrow derived immune cells. It was decided to focus on PROK1 as its expression in the endometrium, as assessed by Northern blot and Taqman quantitative PCR, is suggested to be elevated compared with PROK2 and expression of PROK2 does not change across the menstrual cycle (LeCouter *et al*, 2001, Li M *et al*, 2001, LeCouter *et al*, 2003, Battersby *et al*, 2004a).

PROK1 expression is elevated during the secretory phase of the menstrual cycle (Battersby *et al*, 2004a), and is suggested to be specifically elevated in the mid-secretory phase of the cycle, also known as the window of implantation (Ngan *et al*, 2006). This study confirms the elevated expression of PROK1 in the secretory phase of the menstrual cycle compared with the proliferative phase of the menstrual cycle. However, although PROK1 expression appeared to be further elevated in the mid-secretory phase of the menstrual cycle this was not significant when compared with the early- or late-secretory phases. In agreement with previous data, PROKR1 expression does not appear to exhibit differences between the stages of the menstrual cycle (Battersby *et al*, 2004a). This study also confirms immunolocalisation of PROK1 to the glandular epithelial and stromal compartments of the non-pregnant endometrium with some endothelial cell expression (Battersby *et al*, 2004a). Localisation of PROKR1 in the human endometrium has previously been investigated by in situ hybridisation. In this study, immunolocalisation of PROKR1 expression throughout the menstrual cycle demonstrated expression in the glandular epithelium, the stromal compartment and endothelial cells of the vasculature in the non-pregnant endometrium, similar to that reported previously for in situ hybridisation (Battersby *et al*, 2004a).

Expression of PROK1 and PROKR1 in the pregnant endometrium was also examined by real-time PCR and immunohistochemistry. Expression of both PROK1 and PROKR1 was detected in all samples examined. However, expression was variable and due to insufficient numbers from each of the weeks of gestation (7 - 12 weeks) it was difficult to conclude whether these changes were gestational

age dependent or due to inter-sample variation. Another factor, which must be taken into consideration when attempting to categorise these samples in terms of gestational age, is that the gestational age, as determined by ultrasound, is not accurate and is usually assigned a gestational week plus a number of days (e.g. 7 weeks + 5 days). However, it is difficult to accurately judge early gestations and the surgical termination of pregnancy usually doesn't take place until a week or more after the ultrasound. A sample may therefore be assigned a certain gestational week when in fact it should belong to another (e.g. assigned to week 7 when in fact it should belong to week 8). Although expression of PROK1 and PROKR1 was variable in the pregnant endometrium, when taken collectively, expression of both factors was significantly elevated when compared with non-pregnant endometrium.

Localisation of PROK1 and PROKR1 was also investigated in the pregnant endometrium with expression localised to the glandular epithelium and endothelial cells. This is similar to the non-pregnant endometrium, with extensive expression demonstrated in the decidualised stromal compartment. Further identification of cell populations was performed using cluster designation (CD) markers to identify cells of bone marrow origin and endothelial cells. Macrophages were identified within the pregnant endometrium and were demonstrated to express both PROK1 and PROKR1. However, investigation of uNK cells by PCR and immunohistochemical analysis revealed them to express PROK1, but not PROKR1. A single decidua sample was found to be contaminated with trophoblast tissue, which exhibited immunostaining for both PROK1 and PROKR1. In agreement with the report of Hoffman *et al* (2006), PROK1 localised to the syncytiotrophoblast and cytotrophoblast layers of the invading trophoblast. The study reported herein also shows that PROKR1 localises to these trophoblast cell layers.

In this study, investigation of PROK1 and PROKR1 expression in endometrial cancer revealed no differences between well, moderately or poorly differentiated cancer samples for either PROK1 or PROKR1. A previous study has reported that PROK1 expression is lower in endometrial carcinoma compared with normal endometrium taken during the mid-secretory phase of the menstrual cycle (Ngan

et al, 2006). In this study expression of PROK1 and PROKR1 in endometrial cancer was compared with endometrial samples taken across the proliferative and secretory phases of the cycle rather than solely the mid-secretory phase. Expression of PROK1 in endometrial carcinoma samples appeared to be higher than the level of expression observed in the proliferative phase of the menstrual cycle but was significantly lower than that of the secretory phase. However, PROKR1 expression in endometrial cancer appeared to be similar to the level of expression observed in the proliferative phase of the menstrual cycle. Immunohistochemical analysis of PROK1 and PROKR1 in endometrial cancer revealed little immunostaining for either protein in well, moderately or poorly differentiated samples.

It has been suggested that elevation of PROK1 expression in the secretory phase of the menstrual cycle may be due to regulation by steroid hormones, as PROK1 has a progesterone response element in its promoter region (Battersby *et al*, 2004a). PROK1 expression is also suggested to be subject to regulation by estrogen (Ngan *et al*, 2006). The physiological relevance for this is unclear as during the proliferative phase when estrogen is elevated PROK1 expression is low. PROK1 expression is elevated post ovulation and its expression appears to parallel progesterone secretion. Indeed it has been demonstrated that progesterone induces expression of PROK1 in endometrial explants (Battersby *et al*, 2004a). During the secretory phase of the cycle PROK1 localises to the epithelial and stromal compartments. However, during the secretory phase of the cycle stromal cell expression of the progesterone receptor is elevated while epithelial cell expression is down-regulated (Mote *et al*, 1999, Lessey *et al*, 1996). It is therefore possible that progesterone regulation of PROK1 in the endometrium may be mediated via direct and indirect mechanisms. Progesterone may directly induce expression of PROK1 in the stromal cells where progesterone receptor expression is present. Whereas in epithelial cells, where the receptors are absent, progesterone may mediate PROK1 expression though indirect mechanisms via local paracrine mediators from the stromal cells.

Elevation of PROK1 in the mid-secretory phase of the menstrual cycle, the stage of endometrial receptivity, may suggest a role for PROK1 in preparation of the

endometrium for implantation. PROK1 induces production of nitric oxide (Lin R *et al*, 2002) and induces formation of fenestrae in endothelial cells (LeCouter *et al*, 2001). These actions may contribute to the edema and hyperpermeability observed in the endometrium at the time of implantation (Lessey, 2002). Hyperaemia and endothelial leakage are considered to be some of the first signs of implantation (Plaks *et al*, 2006).

During the secretory phase of the menstrual cycle and early pregnancy the endometrium experiences a large influx of immune cells, mainly uNK cells and macrophages (Loke & King, 1995). The uNK represent over half of the endometrial leukocyte population in the secretory phase of the cycle with their numbers rising further in early pregnancy where they account for around 70% of the leukocytes present (King *et al*, 1998). During the secretory phase and early pregnancy they are found, as demonstrated in Figure 3.8, clustered around the spiral arterioles and glandular epithelium. In pregnancy they are also found thickly clustered around the invading trophoblast (Trundley & Moffett, 2004). uNK cells are thought to play a role in the remodelling of the endometrial vessels upon implantation (Guimond *et al*, 1997). Factors released from the uNK cells, including VEGF (Leonard *et al*, 2006, Li XF *et al*, 2001, Tayade *et al*, 2006, Hanna *et al*, 2006) are thought to potentially act as trophic signals to 'guide' the trophoblast towards the maternal arterioles and guide maternal blood vessel growth towards the invading trophoblast (Carmeliet, 2005). PROK1 has been demonstrated as a chemotactic factor for endothelial cells (Lecouter *et al*, 2001) and may therefore also act as a trophic signal. Upon PROK1-PROKR1 interaction nitric oxide may be generated (Lin R *et al*, 2002). uNK cells expressing PROK1 could potentially interact with PROKR1 on endothelial cells or the trophoblast and produce nitric oxide or other factors, which may influence vascular remodelling or trophoblast invasion and placentation. A deficiency in uNK cells is associated with inadequate vascular remodelling and trophoblast invasion (Ashkar *et al*, 2003). PROK1 expression in uNK may therefore contribute to this remodelling and placentation.

A role for PROK1 and PROKR1 has been suggested in the immune response and in the survival and differentiation of granulocytic and monocytic cells (Dorsch *et al*, 2005, LeCouter *et al*, 2004). Expression of PROK1 and PROKR1 in

macrophages may imply a role for these proteins in their activation. It is suggested that macrophages may act in a synergistic manner to control each other's activation and the prokineticin system may play a role in this regulation as prokineticins appear to control macrophage activation (Martucci *et al*, 2005). Chemokines produced by the immune cells within the endometrium are thought to promote further recruitment of immune cells into the endometrium (Salamonsen *et al*, 2002, Thelen *et al*, 2001). It is possible that PROK1 or products derived from PROK1-PROKR1 interaction may promote this recruitment during the secretory phase of the cycle and may account, in part, for the dramatic increase in immune cell numbers in the secretory phase and into early pregnancy.

In this study, expression of PROK1 and PROKR1 was investigated in endometrial cancer since it was suggested that PROK1 may play a role in normal and pathological angiogenesis (LeCouter *et al*, 2001, LeCouter *et al*, 2003). However, expression of PROK1 and PROKR1 was not dramatically altered compared with normal endometrium. Indeed, in the case of PROK1 expression the level in endometrial cancer was significantly down-regulated compared with the secretory phase of the menstrual cycle. Hence these factors seem unlikely to play a role in endometrial angiogenesis.

PROK1 is elevated in the non-pregnant endometrium at a time when it is undergoing preparation for pregnancy. PROK1 and its receptor PROKR1 are further elevated in the event of pregnancy where localisation to discrete immune cells is evident. Their signalling, action, target gene regulation and mediators of their expression however are currently unknown or incompletely understood in the endometrium. The PROK1-PROKR1 system clearly warrants further investigation and the remainder of this thesis is dedicated to answering some of these questions to decipher potential roles for PROK1 and PROKR1 in endometrial physiology.

Chapter 4 - Establishment of Ishikawa endometrial cell line stably expressing PROKR1 and characterisation of PROK1 induced signalling in PROKR1 Ishikawa cells

4.1. Introduction

Prokineticin 1 (PROK1) and prokineticin receptor 1 (PROKR1) are expressed in the endometrium and first trimester decidua as described in chapter 3. Signalling induced by PROK1-PROKR1 has been investigated in primary adrenal cortex endothelial (ACE) cells and cell lines transfected with PROKR1. However, to date there has been no characterisation of the signalling downstream of PROK1-PROKR1 in the endometrium.

In the paper which first described the existence of human prokineticins, it was suggested that their receptors were G-protein coupled receptors (GPCR's) due to displacement of their specific binding by GTPγS (Li M *et al*, 2001). The signalling activated by PROK1 binding to an uncharacterised receptor was subsequently investigated in ACE cells. R Lin *et al* (2002), demonstrated elevation of ERK 1/2 phosphorylation in response to treatment with PROK1 in ACE cells. However, the receptors had not been characterised as GPCR's, receptor tyrosine kinases (RTK'S) or others. VEGF and PROK1 induce similar effects on ACE cells, acting as chemo-attractants and inducing formation of fenestrae (LeCouter *et al*, 2001). It was therefore presumed that PROK1 and VEGF might activate the same receptor in ACE cells. In order to investigate whether this was the case, PROK1 was pre-incubated with a soluble VEGF receptor prior to addition to ACE cells. Pre-incubation of PROK1 with a soluble VEGF receptor however did not abrogate the PROK1 induced ERK 1/2 response. The VEGF receptors are RTK's, therefore in order to examine whether the PROK1 receptor was also an RTK the phosphotyrosine status of the ACE cells was examined after stimulation with PROK1. ACE cells did not reveal any elevation in tyrosine phosphorylation after PROK1 stimulation suggesting that the PROK1 receptor is not an RTK in ACE cells (R Lin *et al*, 2002). However, incubation of ACE cells with pertussis toxin, an inhibitor of Gi coupled GPCR's, almost completely abolished the PROK1 mediated ERK 1/2 phosphorylation. These data therefore suggested, in agreement with the report of Li *et al* (2001) that prokineticin receptors were GPCR's.

Three other reports published in 2002 also proposed prokineticin receptors to be GPCR's (Lin DC *et al*, 2002, Masuda *et al*, 2002, Soga *et al*, 2002). In the report of DC Lin *et al* (2002), the prokineticin receptors were characterised as two G-protein coupled receptors GPR73a (or prokineticin receptor 1, PROKR1) and GPR73b (or prokineticin receptor 2, PROKR2). These studies however, demonstrated prokineticin receptors to be Gq coupled rather than Gi coupled (Lin DC *et al*, 2002, Masuda *et al*, 2002, Soga *et al*, 2002). Measurements of Gq activation including inositol phosphate mobilisation (Lin DC *et al*, 2002), and calcium mobilisation (Masuda *et al*, 2002) were used to characterise the receptors. PROK1 and PROK2 are suggested to activate both PROKR1 and PROKR2. This was perhaps most elegantly demonstrated by the ERK phosphorylation data of DC Lin *et al* (2002). They demonstrate maximal ERK 1/2 phosphorylation activated by PROK1 binding to PROKR1, whereas PROK2 activates maximal ERK 1/2 phosphorylation in cells expressing PROKR2. Both ligands, however, were able to activate some level of ERK 1/2 phosphorylation at both receptors.

PROK1 expression is elevated compared with PROK2 in the endometrium and demonstrates cyclical variation across the menstrual cycle (LeCouter *et al*, 2001, Li M *et al*, 2001, Battersby *et al*, 2004a). PROK1 also preferentially activates PROKR1 (Lin DC *et al*, 2002). This thesis was therefore directed towards further understanding the role of PROK1-PROKR1 in the human endometrium. An endometrial epithelial cell line stably expressing PROKR1 was generated, and downstream signalling pathways activated upon PROK1-PROKR1 interaction subsequently analysed.

4.2. Materials and methods

4.2.1. Transfection of PROKR1 cDNA

This was fully described in Section 2.3. Briefly, human cDNA encoding prokineticin receptor 1 (PROKR1) was amplified using RT-PCR. The cDNA was amplified using Easy-A DNA polymerase (Stratagene) and specific primers:

Forward 5′ GGA TCC AAG CTT GAT GGA GAC CAC CAT GGG G 3′

Reverse 5′ CTC GAG GAT ATC TTA TTT TAG TCT GAT GCA GTC CAC CT 3′.

The PCR product was excised from a 1% agarose gel following electrophoresis, purified, ligated into sequencing vector pCR4 (Invitrogen) and cloned into E.Coli TOP10 cells. Cloned plasmid DNA was subjected to automated DNA sequencing to confirm the cDNA sequence prior to subcloning the Hind III-EcoRV fragment into pcDNA 3.1(+). This expression construct was transfected into Ishikawa cells using electroporation and a G418 resistant clone isolated. These cells are subsequently termed PROKR1 Ishikawa cells.

4.2.2. Polymerase chain reaction

RNA was extracted from WT and PROKR1 Ishikawa cells as described in Section 2.5. Briefly, media was removed from cells and TRI-reagent added. RNA was extracted by the phenol chloroform method. RNA was quantified and prepared at a concentration of 1μg in a volume of 10μl in order to make cDNA as described in Section 2.5. In order to amplify PROKR1 cDNA, PCR was performed using specific PROKR1 primers provided in section 4.2.1. A 1% TAE agarose gel was prepared and samples run for approximately 1 hour 30 minutes before visualisation using a UV transilluminator.

4.2.3. Taqman quantitative PCR analysis

Expression of RNA within WT and PROKR1 Ishikawa cells was examined by Taqman quantitative PCR analysis as described in Section 2.5. Briefly, cDNA was prepared in a random hexamer primed reaction using 200ng RNA per reaction. Quantitative PCR analysis was performed on the cDNA using specific primer probe combinations to detect PROKR1 (sequence given in Table 5). The probe was given a FAM fluorescent label with a TAMRA quencher. Gene expression was normalized by including Vic labelled primer-probe mix to detect the 18s ribosomal subunit (sequence given in Table 5), as a loading control for the amount of cDNA added in each sample.

Reaction mixes were loaded in duplicate onto a 96-well MicroAmp fast optical reaction plate for analysis on an ABI7900 HT Fast Real-Time PCR machine. Data were analysed and processed using sequence detector version 1.6.3. Results were expressed as relative to a positive RNA standard included in all

reactions. The data were analysed using the comparative C_T method for relative quantification.

4.2.4. Immunocytochemistry

2 x10^5 PROKR1 and WT Ishikawa cells were seeded in 2 well chamber slides, allowed to attach overnight, subsequently washed in PBS and fixed in ice cold methanol. A peroxidase block was applied followed by a 5% normal serum block (swine) prior to application of rabbit anti-PROKR1 (1:500) overnight at 4°C. The cells were washed in PBS-tween and PBS, followed by application of swine anti-rabbit peroxidase labelled antibody (1:200) for 2 hours at room temperature. TyrCy3 Tyramide amplification agent in substrate was subsequently applied for 10 minutes. Nuclear counterstain DAPI (1:1000) was applied prior to mounting in Permafluor aqueous mounting agent. Fluorescent images were visualised using a laser scanning confocal microscope (LSM 510, Carl Zeiss, Jena, Germany).

4.2.5. IP assay

PROKR1 Ishikawa cells were seeded out in 12-well plates to a density of 100,000 cells/well and left to settle for 24 hours. The cells were then incubated in special inositol free DMEM with 1μCi/ml [^{3}H] myo-inositol for 24 hours. In order to determine the concentration of PROK1 which induced the greatest Inositol phosphate mobilisation the cells were subsequently stimulated with vehicle, 4pM, 40pM, 400pM, 4nM and 40nM PROK1 for one hour. PROK1 stimulated reactions were terminated by removal of agonist followed by addition of ice cold 10nM formic acid for at least 45 minutes at 4°C. Supernatants were then loaded into tubes containing AG-1X8 resin. Samples were washed a number of times as indicated in section 2.6. 1M ammonium formate/0.1M formic acid was added to the resin, vortexed and supernatant removed to scintillation vials. Scintillation fluid was added and ^{3}H counted as indicated in Section 2.6.

4.2.6. cAMP assay

PROKR1 Ishikawa cells were seeded out in 3cm cell culture dishes to a density of 200,000 cells/dish and left to settle for 24 hours. Subsequently the cells

were incubated in serum free medium overnight prior to stimulation. The phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) was added to the cells at a final concentration of 1mM for 30 minutes at 37°C prior to treatment with PROK1. 40nM PROK1 was added to the medium for 0, 5, 10, 15 or 20 minutes. Additionally, treatment with Forskolin (10μM) for 10 minutes was used as a positive control. To terminate the reaction the cell culture medium was removed and assayed for cAMP as described in Section 2.10. The cells were lysed in hydrochloric acid (HCl) and assayed for protein concentration using the modified Bradford method as described in Section 2.7. Concentration of cAMP within samples was normalised for protein concentration.

4.2.7. PROK1 mediated signalling

4.2.7.1. PROK1 dose response of ERK 1/2 phosphorylation

A dose response study was conducted in order to investigate PROK1 induced ERK 1/2 phosphorylation in PROKR1 Ishikawa cells and to determine the concentration of PROK1 which elicited maximal ERK 1/2 phosphorylation. PROKR1 Ishikawa cells were seeded at a density of 250,000 cells/well in a 6-well plate. PROKR1 Ishikawa cells were subjected to serum starvation overnight followed by stimulation with 4pM, 40pM, 400pM, 4nM or 40nM PROK1 for 5 minutes. Reactions were terminated by removal of agonist and cells were washed in ice cold PBS. The cells were lysed in NP40 lysis buffer and processed for Western immunoblot analysis, as indicated in Section 4.2.7.

4.2.7.2. Time course of PROK1 induced ERK 1/2 phosphorylation

A time course study was conducted in order to determine the time point at which 40nM PROK1 induced maximal ERK 1/2 phosphorylation. PROKR1 Ishikawa cells were seeded at a density of 200,000 cells in 3cm dishes. PROKR1 Ishikawa cells were subjected to serum starvation overnight followed by stimulation with 40nM PROK1 for 0, 1, 5, 10 and 20 minutes. Reactions were terminated by removal of agonist and cells were washed in

ice cold PBS. The cells were lysed in NP40 lysis buffer and processed for Western immunoblot analysis, as indicated in Section 4.2.7.

4.2.7.3. Effect of chemical inhibitors of signalling intermediates on PROK1 induced ERK 1/2 phosphorylation

In order to determine the signalling molecules activated by PROK1 in the signalling cascade to ERK 1/2 phosphorylation chemical inhibitors of signalling molecules were employed. PROKR1 Ishikawa cells were seeded at a density of 250,000 cells/well in a 6-well plate. PROKR1 Ishikawa cells were subjected to serum starvation overnight followed by pre-incubation with chemical inhibitors or vehicle for one hour prior to stimulation with 40nM PROK1 for 5 minutes. The chemical inhibitors and their working concentrations are summarised in Table 9. Reactions were terminated by removal of agonist and cells were washed in ice cold PBS. The cells were lysed in NP40 lysis buffer and processed for Western immunoblot analysis, as indicated in Section 4.2.7.

Table 9. Concentrations of inhibitors of signalling molecules

Inhibitor	Molecule Inhibited	Inhibitor concentration
YM254890	Gq protein	1μM
Pertussis Toxin	Gi protein	200ng/ml
U73122	Phospholipase C	10μM
BAPTA-AM	Calcium	50μM
PP2	cSrc	10μM
AG1478	EGFR	200nM
PD98059	MEK	50μM

4.2.7.4.Effect of dominant negative isoforms of signalling molecules on PROK1 induced ERK 1/2 phosphorylation

In order to further investigate the signalling molecules involved in PROK1 signalling to ERK 1/2, dominant negative isoforms of signalling molecules were used. These have been previously characterized and described (Harris, 2002, Levi, 1998). PROKR1 Ishikawa cells were seeded to a density of 500,000 cells in a 6cm dish. PROKR1 Ishikawa cells were transiently co-transfected with 2.5μg c-Myc ERK and 7.5μg empty vector

(pcDNA3) or dominant negative isoforms of cSrc, EGFR, MEK or the small GTPase Ras in the presence of Superfect transfection reagent for 6 hours. Transfection mix was removed and cells incubated in complete DMEM Glutamax media. Transiently transfected PROKR1 Ishikawa cells were subjected to serum starvation overnight followed by stimulation with 40nM PROK1 for 5 minutes. One sample transfected with pcDNA3 was incubated with Gq protein inhibitor YM254890 for one hour prior to stimulation with 40nM PROK1 for 5 minutes. Reactions were terminated by removal of agonist and cells were washed in ice cold PBS. The cells were lysed in NP40 lysis buffer and immunoprecipitated with Myc-ERK agarose conjugate antibody overnight with rotation at 4°C. The immunoprecipitated samples were collected by centrifugation at 14000 rpm for 15 minutes and washed in ice-cold NP40 lysis buffer 3 times. Samples were resuspended in Laemmli protein gel loading buffer (Section 2.12) and processed for Western immunoblot analysis, as indicated in Section 4.2.7.

4.2.7.5. PROK1 induced cSrc and EGFR phosphorylation

A time course study was conducted in order to determine the time point at which PROK1 induced maximal cSrc and EGFR phosphorylation. PROKR1 Ishikawa cells were seeded at a density of 3 million cells in 10cm dishes. PROKR1 Ishikawa cells were subjected to serum starvation overnight followed by stimulation with 40nM PROK1 for 0, 1, 5, 10 and 20 minutes. Reactions were terminated by removal of agonist and cells were washed in ice cold PBS. The cells were lysed in NP40 lysis buffer and immunoprecipitated with anti-phosphotyrosine (PY20) agarose conjugate to immunoprecipitate the tyrosine phosphorylated cSrc or EGFR. The immunoprecipitated samples were collected by centrifugation at 14000 rpm for 15 minutes and washed in ice-cold NP40 lysis buffer 3 times. Samples were resuspended in Laemmli protein gel loading buffer (Section 2.12) and processed for Western immunoblot analysis, as indicated in Section 4.2.7.

4.2.8. Western immunoblot analysis

Cells were lysed in NP40 lysis buffer (described in Sections 2.7 and 2.12) and protein concentration assayed as described in Section 2.7. Proteins were solubilised in Laemmli protein gel loading buffer (Section 2.12). Samples were boiled for 5 minutes in order to denature proteins prior to resolution on an SDS-PAGE gel as described in Sections 2.7.2 and 2.7.3. Proteins were immunoblotted to a PVDF membrane and non-specific binding blocked with blocking buffer. Immobilised proteins on the PVDF membrane were probed with specific primary antibodies for phosphorylated ERK 1/2, total ERK 1/2, phosphorylated cSrc or phosphorylated EGFR (antibody dilutions in Table 10). Immunoblotted proteins were visualised by a chemifluorescent or direct fluorescent detection system as indicated in Section 2.7.

Table 10. Antibody dilutions used for Western immunoblot analysis

Antibody	Species raised in	Dilution	Purchased from
pERK	Rabbit	1:1000	Cell Signalling
tERK	Mouse	1:1000	Cell signalling
p-EGFR	Goat	1:500	Santa Cruz/ Autogen Bioclear
cSrc	Rabbit	1:500	Santa Cruz/ Autogen Bioclear

4.2.9. Statistics

Statistical analysis of the data in this study was carried out by ANOVA with Fishers PLSD (predicted least squares difference) applied, using Statview 5.0 (Abacus Concepts). The data is presented as mean ± SEM of at least 3 experiments.

4.3. Results

4.3.1. Production of PROKR1 Ishikawa cell line

Transfection of PROKR1 cDNA into an endometrial epithelial cell line was performed in order to provide a model system with which to investigate PROK1-PROKR1 induced signalling. One clone was isolated and subsequently screened to assess the level of PROKR1 expression compared with WT Ishikawa cells.

Semi-quantitative and real-time quantitative PCR was performed on RNA isolated from WT and PROKR1 Ishikawa cells. Semi-quantitative PCR analysis of PROKR1 expression in WT and PROKR1 Ishikawa cells revealed a single band of the expected molecular weight in PROKR1 Ishikawa cells (Figure 4.1 A). No band was evident in WT Ishikawa cells. Taqman quantitative PCR analysis of PROKR1 expression in PROKR1 Ishikawa cells compared with WT Ishikawa cells confirmed these data. Minimal PROKR1 mRNA expression was detected in WT Ishikawa cells whereas PROKR1 was found to be expressed in PROKR1 Ishikawa cells (Figure 4.1 B).

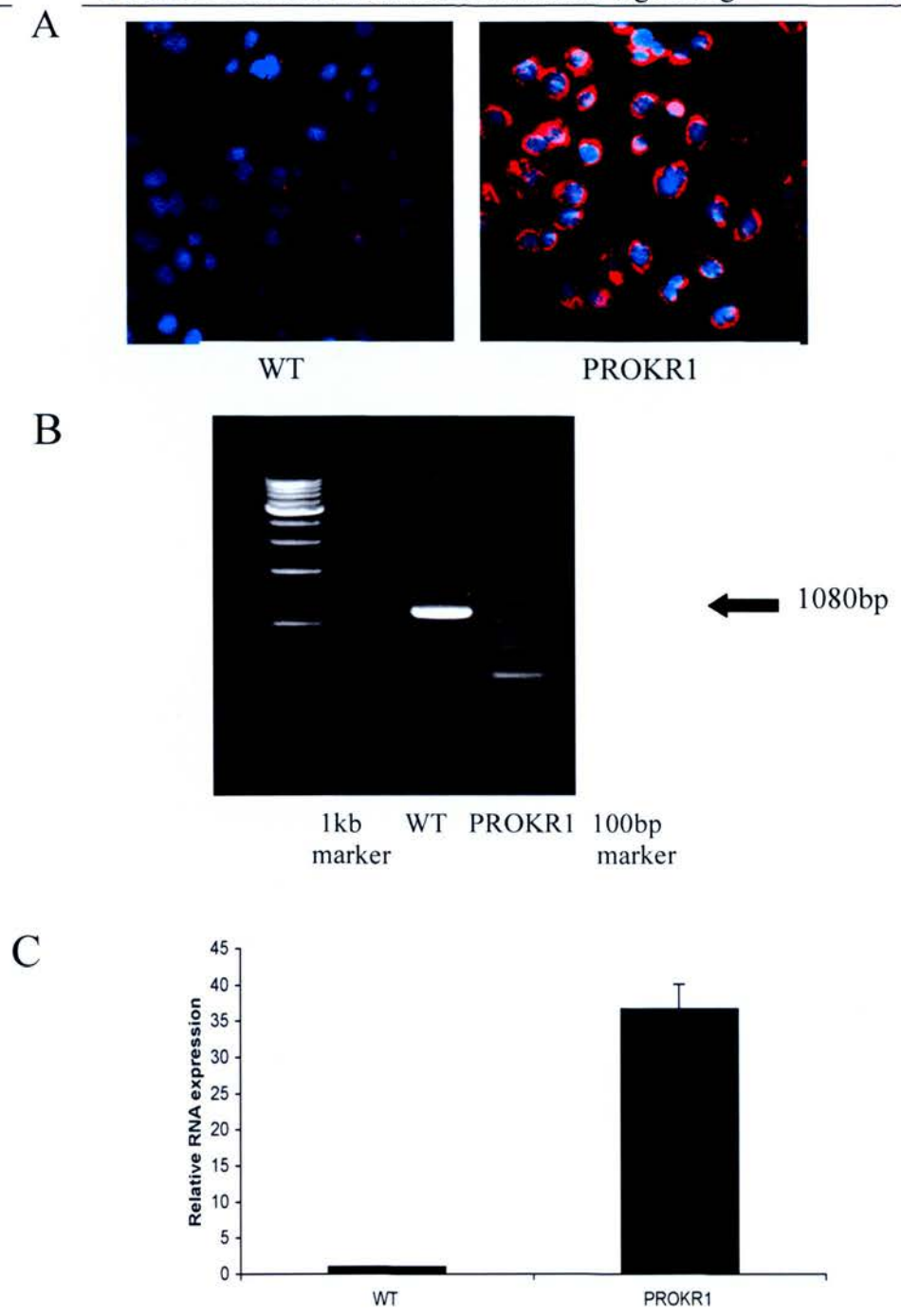


Figure 4.1. Immunocytochemistry, semi quantitative and real-time Taqman quantitative PCR expression of PROKR1 in WT and PROKR1 Ishikawa cells. A. Immunocytochemistry indicates staining for PROKR1 in PROKR1 Ishikawa cells (red) but not in WT Ishikawa cells. B. PCR gel reveals a band at the expected molecular weight size of 1080bp in PROKR1 Ishikawa cells; no band was evident in WT Ishikawa cells. C. PROKR1 and WT Ishikawa cells were examined for expression of PROKR1 by Taqman quantitative PCR analysis. PROKR1 expression was normalised for loading against expression of 18s and relative to a single endometrial control. Data are presented as mean ± SEM.

4.3.2. Signalling characterisation of PROKR1 Ishikawa cell line

4.3.2.1. PROK1 mediated inositol phosphate mobilisation

Prokineticin has been suggested to couple to Gq in HEK293 and CHO cells transfected with prokineticin receptors (Lin DC *et al*, 2002, Masuda *et al*, 2002, Soga *et al*, 2002). In order to investigate if PROKR1 couples to Gq proteins in PROKR1 Ishikawa cells and demonstrate the presence of functional prokineticin receptors in PROKR1 Ishikawa cells, inositol phosphate mobilisation was measured following treatment with PROK1. WT and PROKR1 Ishikawa cells were stimulated with vehicle, 4pM, 40pM, 400pM, 4nM and 40nM PROK1 for one hour. No significant mobilisation of inositol phosphate was detected in WT Ishikawa cells at any dose of PROK1 used (Figure 4.3, grey bars). However, significant elevation of inositol phosphate mobilisation was detected upon treatment of PROKR1 Ishikawa cells with 40nM PROK1 (2.71 ± 0.25 fold above vehicle treated control, $P<0.05$, Figure 4.3, black bars). No significant elevation in inositol phosphate mobilisation was noted in PROKR1 Ishikawa cells at lower concentrations. These data indicate that PROKR1 may be Gq coupled in PROKR1 Ishikawa cells.

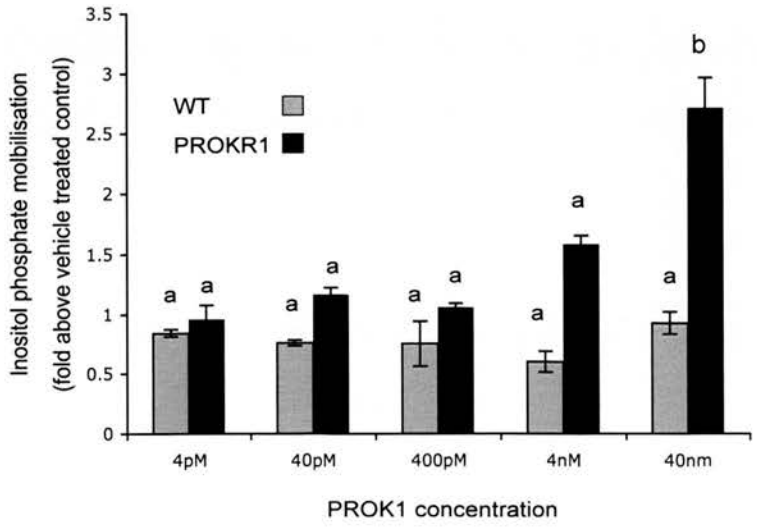


Figure 4.2. Induction of Inositol phosphate mobilisation in WT and PROKR1 Ishikawa cells. WT and PROKR1 Ishikawa cells were stimulated with vehicle, 4pM, 40pM, 400pM, 4nM and

40nM PROK1 for 1 hours followed by assay for inositol phosphate. The data are presented as mean ± SEM. (b is significantly different from a P<0.05).

4.3.2.2. PROK1 mediated cAMP production

In order to investigate whether PROKR1 can couple to Gs proteins, production of cAMP in PROKR1 Ishikawa cells was measured following treatment with PROK1. PROKR1 Ishikawa cells were treated with 40nM PROK1 for 0, 5, 10, 15 or 20 minutes or forskolin for 10 minutes. No significant production of cAMP was detected in response to PROK1 treatment (Figure 4.2). However cAMP production was elevated following treatment with forskolin for 10 minutes (362.85±52.15 pmol/mg protein). Forskolin is known to induce cAMP release (Seamon *et al*, 1981). These data demonstrate that PROKR1 is not Gs coupled in PROKR1 Ishikawa cells.

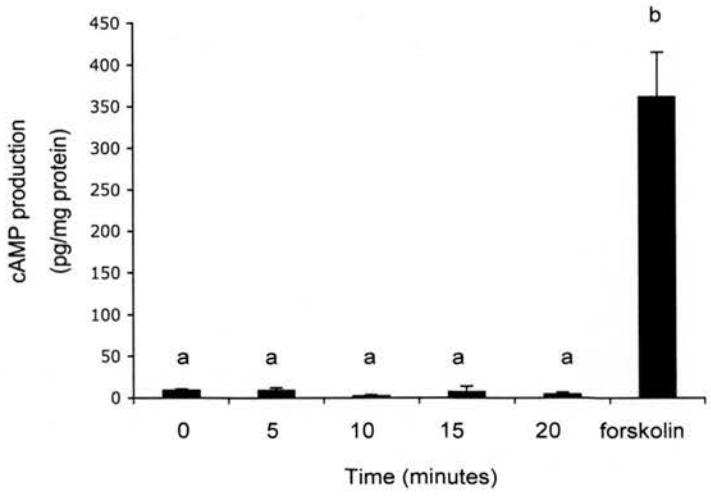


Figure 4.3. Induction of cAMP production in response to 40nM PROK1 and forskolin in PROKR1 Ishikawa cells. PROKR1 Ishikawa cells were stimulated with 40nM PROK1 for 0, 1, 5, 10 and 20 minutes. Forskolin treatment was applied for 10 minutes. The media was assayed for cAMP by ELISA. The data are presented as mean ± SEM (b is significantly different from a p<0.001).

4.3.2.3.Dose response of PROK1 induced ERK 1/2 phosphorylation

Activation of PROKR1 has been demonstrated to induce ERK 1/2 phosphorylation (Lin R *et al*, 2002, Lin DC *et al*, 2002). In order to further investigate the signalling pathways activated upon PROKR1-PROK1 activation in PROKR1 Ishikawa cells, ERK 1/2 phosphorylation was

examined. PROKR1 Ishikawa cells were treated with vehicle, 4pM, 40pM, 400pM, 4nM and 40nM PROK1 for 5 minutes and ERK 1/2 phosphorylation examined by Western immunoblot analysis. ERK 1/2 phosphorylation was significantly elevated upon treatment with 40nM PROK1 at 5 minutes (5.21 ± 0.52 fold above vehicle treated control, $P<0.01$, Figure 4.4) compared with all other doses of PROK1. Hence 40nM PROK1 was used for all subsequent experiments.

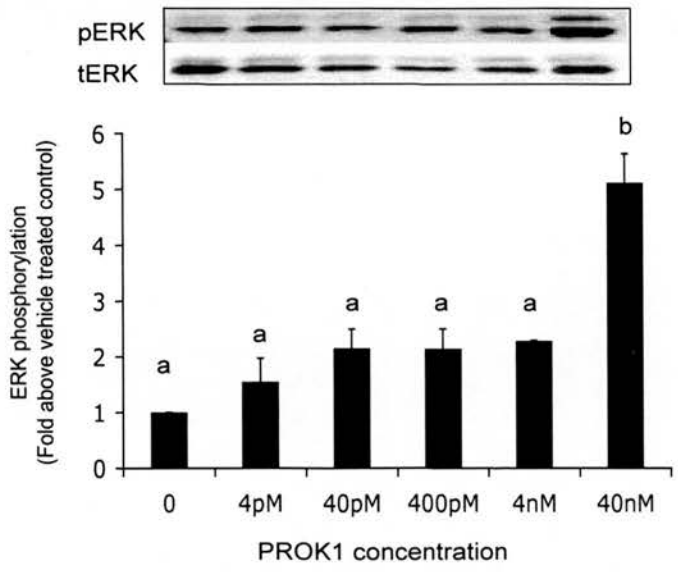


Figure 4.4. Induction of ERK 1/2 phosphorylation in PROKR1 Ishikawa cells. PROKR1 Ishikawa cells were stimulated with vehicle, 4pM, 40pM, 400pM, 4nM and 40nM PROK1 for 5 minutes. Proteins were extracted, quantified and 20μg resolved by SDS-PAGE followed by Western immunoblot analysis using specific antibodies against phosphorylated ERK 1/2 (pERK). The total amount of ERK in cell lysates was determined by probing the same blot with antibody recognising total ERK 1/2 (tERK). ERK 1/2 phosphorylation was normalised for tERK expression. Data are expressed as fold above vehicle treated control. A representative Western immunoblot is shown with semi-quantitative densitometric analysis of ERK 1/2 phosphorylation. The data are presented as mean ± SEM. (b is significantly different from a $P<0.01$).

4.3.2.4.Time course of PROK1 induced ERK 1/2 phosphorylation

Maximal phosphorylation of ERK 1/2 via PROKR1-PROK1 was observed following treatment with 40nM PROK1. Subsequently the time of maximal ERK 1/2 phosphorylation in response to 40nM PROK1 was investigated. PROKR1 Ishikawa cells were treated with 40nM PROK1 for 0, 1, 5, 10 and

20 minutes and ERK 1/2 phosphorylation determined by Western immunoblot analysis. ERK 1/2 phosphorylation peaked at 5 minutes (8.03 ± 1.59 fold above vehicle treated control, P<0.001, Figure 4.5) with significant elevation sustained to 10 minutes (7.79 ± 1.54 fold above vehicle treated control, P<0.001, Figure 4.5). Five minutes stimulation with 40nM PROK1 was used for all subsequent ERK 1/2 phosphorylation investigations in PROKR1 Ishikawa cells.

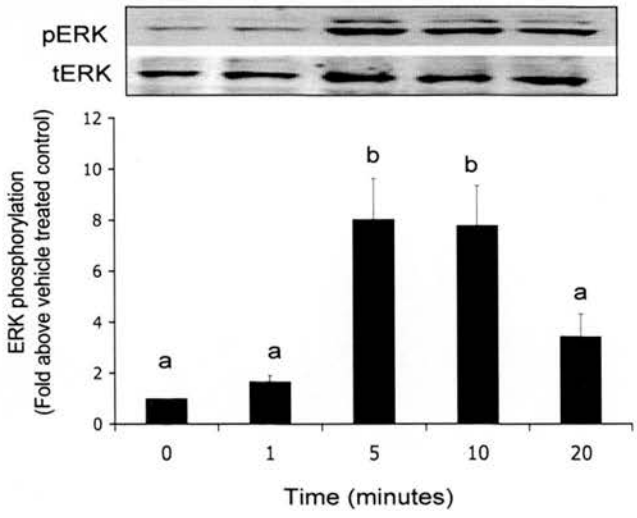


Figure 4.5. Time course of PROK1 induced ERK 1/2 phosphorylation. Induction of ERK 1/2 phosphorylation in PROKR1 Ishikawa cells stimulated with 40nM PROK1 for 0, 1, 5, 10 and 20 minutes. Proteins were extracted, quantified and 20μg resolved by SDS-PAGE followed by Western immunoblot analysis using specific antibodies against phosphorylated ERK 1/2 (pERK). The total amount of ERK in cell lysates was determined by probing the same blot with antibody recognising total ERK 1/2 (tERK). ERK 1/2 phosphorylation was normalised for tERK expression. Data are expressed as fold above vehicle treated control. A representative Western immunoblot is shown with semi-quantitative densitometric analysis. The data are presented as mean ± SEM. (b is significantly different from a P<0.001).

4.3.3. Mapping the pathway of PROK1-PROKR1 induced signalling to ERK 1/2

4.3.3.1. PROKR1 signalling is not Gi coupled

It has previously been suggested that PROKR1 couples to either Gq or Gi (Lin R *et al*, 2002, Lin DC *et al*, 2002, Masuda *et al*, 2002, Soga *et al*, 2002). In PROKR1 Ishikawa cells, PROK1 induces inositol phosphate

mobilisation, suggesting that in this model system PROKR1 couples to Gq. However, pertussis toxin (PTX) a powerful inhibitor of Gi coupling was used in order to rule out coupling of PROKR1 to Gi. PROKR1 cells were pre-incubated with 200ng/ml PTX overnight or for one hour prior to stimulation with 40nM PROK1 for 5 minutes. PTX did not inhibit PROK1 induced ERK 1/2 phosphorylation with overnight pre-treatment or pre-treatment for 1 hour. These data demonstrate that PROKR1 does not couple to Gi in PROKR1 Ishikawa cells.

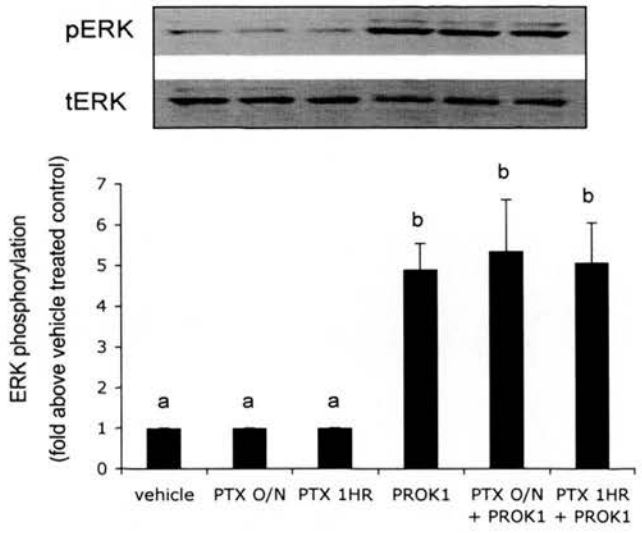


Figure 4.6. ERK 1/2 phosphorylation is not inhibited by PTX. PROKR1 Ishikawa cells were pre-treated with vehicle or 200ng/ml PTX overnight or for one hour prior to stimulation with 40nM PROK1. Proteins were extracted, quantified and 20μg resolved by SDS-PAGE followed by Western immunoblot analysis using specific antibodies against phosphorylated ERK 1/2 (pERK). The total amount of ERK in cell lysates was determined by probing the same blot with antibody recognising total ERK 1/2 (tERK). ERK 1/2 phosphorylation was normalised for tERK expression. Data are expressed as fold above vehicle treated control. A representative Western immunoblot is shown with semi-quantitative densitometric analysis. The data are presented as mean ± SEM. (b is significantly different from a $P<0.001$).

4.3.3.2.PROKR1 is Gq coupled and ERK 1/2 phosphorylation is mediated via PLC-β - Ca^{2+} - cSrc – EGFR and MEK

Phosphorylation of ERK 1/2 following activation of GPCR's is dependent on activation of intracellular signalling-phosphorylation cascades (Werry *et al*, 2005). Hence, the cascade of signalling molecules activated by PROK1

signalling to ERK 1/2 in PROKR1 Ishikawa cells was investigated. PROKR1 is a Gq coupled receptor in PROKR1 Ishikawa cells, as demonstrated by mobilisation of inositol phosphate. To confirm this, PROKR1 Ishikawa cells were treated with 40nM PROK1 for 5 minutes in the presence or absence of a specific inhibitor of Gq protein (YM254890) in addition to inhibitors of phospholipase C-β (U73122), calcium (BAPTA-AM), cSrc (PP2), EGFR (AG1478) and MEK (PD98059). ERK 1/2 phosphorylation in response to PROK1 (5.86 ± 0.58 fold above vehicle treated control, Figure 4.7, lane 2, $p<0.001$) was abolished by co-treatment with Gq inhibitor (Figure 4.7, lane 3), PLC-β inhibitor (Figure 4.7, lane 4), Ca^{2+} inhibitor (Figure 4.7, lane 5), cSrc inhibitor (Figure 4.7, lane 6), EGFR inhibitor (Figure 4.7, lane 7) and MEK inhibitor (Figure 4.7, lane 8). These data demonstrate that PROK1 induces ERK 1/2 phosphorylation in PROKR1 Ishikawa cells via a Gq coupled pathway involving activation of multiple signalling molecules including the non-RTK cSrc and RTK EGFR.

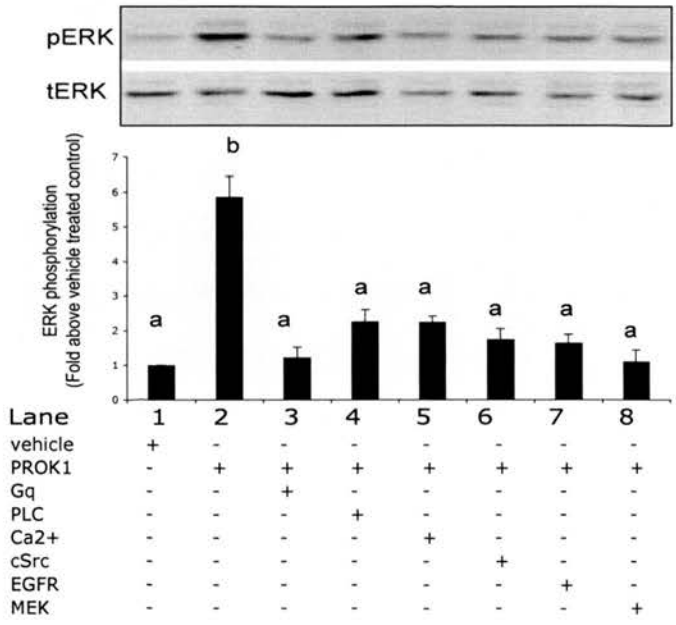


Figure 4.7. Chemical inhibitors of signalling intermediates inhibit PROK1 induced ERK 1/2 phosphorylation. PROKR1 Ishikawa cells were treated with 40nM PROK1 in the presence or absence of inhibitors of Gq, PLC, Ca^{2+}, cSrc, EGFR and MEK. Proteins were extracted, quantified and 20μg resolved by SDS-PAGE followed by Western immunoblot analysis using specific antibodies against phosphorylated ERK 1/2 (pERK). The total amount of ERK in cell lysates was determined by probing the same blot with antibody recognising total ERK 1/2

(tERK). ERK 1/2 phosphorylation was normalised against tERK expression. Data are expressed as fold above vehicle treated control. PROK1 mediated ERK 1/2 phosphorylation was significantly inhibited by pre-treatment with inhibitors of Gq protein (YM254890, 1μM, lane 3), PLC (U73122, 10μM, lane 4), Ca^{2+} (BAPTA-AM, 50μM, lane 5), cSrc (PP2, 10μM, lane 6), EGFR (AG1478, 200nM, lane 7) and MEK (PD98059, 50μM, lane 8). A representative Western immunoblot is shown with semi-quantitative densitometric analysis. + denotes presence of agent, - denotes absence of agent. The data are presented as mean ± SEM. (b is significantly different from a $P<0.001$).

4.3.3.3. PROK1 mediated ERK 1/2 phosphorylation is inhibited by dominant negative isoforms of signalling pathway intermediates

In order to confirm a role for cSrc, EGFR, MEK and establish a role for the small monomeric G-protein Ras (against which there are no reliable chemical inhibitors) cDNA constructs encoding dominant negative mutant isoforms of the above signalling molecules were utilised. PROKR1 Ishikawa cells were transiently transfected with Myc-tagged ERK and empty vector cDNA (pcDNA3) or cDNA encoding dominant negative mutants of cSrc, EGFR, Ras and MEK. Cells were subsequently treated with 40nM PROK1 for 5 minutes, lysed and immunoprecipitated using Myc-tagged ERK antibodies followed by Western immunoblot analysis for ERK 1 phosphorylation. PROK1 induced phosphorylation of Myc-tagged ERK (5.36 ± 1.37 fold above vehicle treated control Figure 4.8, lane 2, $p<0.001$) was abolished by co-expression of the dominant negative isoforms of cSrc (Figure 4.8, lane 4), EGFR (Figure 4.8, lane 5), Ras (Figure 4.8, lane 6) and MEK (Figure 4.8, lane 7) with Myc-ERK. These data confirm that ERK 1 phosphorylation is dependent on activation of cSrc and EGFR as well as the small monomeric G-protein Ras.

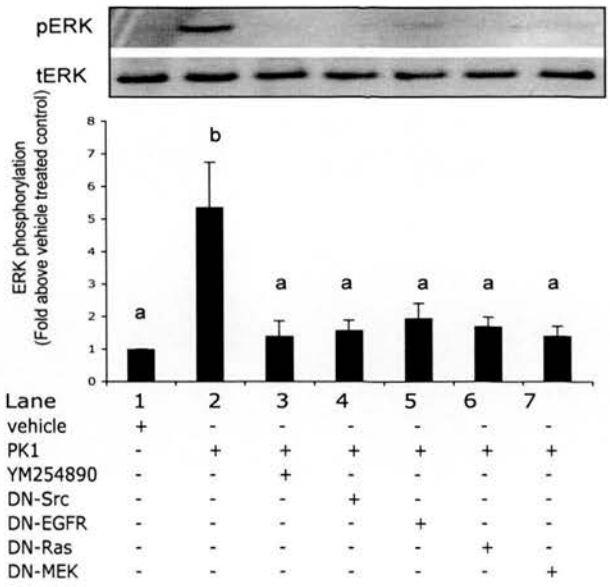


Figure 4.8. Inhibition of ERK 1/2 phosphorylation using cDNA constructs encoding dominant negative mutant isoforms of cSrc, EGFR, Ras and MEK. PROKR1 Ishikawa cells were co-transfected with Myc-tagged ERK and either empty vector (pcDNA3) or dominant negative isoforms of cSrc, EGFR, Ras and MEK prior to stimulation with 40nM PROK1 for 5 minutes. 1mg of protein was immunoprecipitated using Myc-tagged ERK agarose conjugated antibody. Protein was resolved by SDS-PAGE followed by Western immunoblot analysis using specific antibodies against phosphorylated ERK 1/2 (pERK). The total amount of ERK in cell lysates was determined by probing the same blot with antibody recognising total ERK 1/2 (tERK). ERK 1/2 phosphorylation was normalised against tERK expression. Data are expressed as fold above vehicle treated control. Co-transfection of PROKR1 Ishikawa cells with c-Myc tagged ERK and dominant negative isoforms of cSrc, EGFR, Ras or MEK significantly inhibited PROK1 induced ERK phosphorylation. + denotes presence of agent, - denotes absence of agent. A representative Western immunoblot is shown with semi-quantitative densitometric analysis. The data are presented as mean ± SEM. (b is significantly different to a $P<0.001$).

4.3.3.4.PROK1 induces phosphorylation of cSrc

Studies using chemical inhibitors and cDNA constructs encoding dominant negative mutants of signalling molecules suggested a role for the non-receptor tyrosine kinase cSrc in mediating ERK 1/2 phosphorylation. cSrc phosphorylation in response to PROK1 stimulation was examined using specific antisera, which recognises cSrc Tyr418, the stabilised form of the cSrc kinase domain. PROKR1 Ishikawa cells were stimulated with 40nM PROK1 for 0, 1, 5, 10 and 20 minutes and immunoprecipitated with anti-phosphotyrosine (PY20) agarose conjugate to immunoprecipitate the

tyrosine-phosphorylated cSrc. Immunoprecipitated proteins were subjected to Western immunoblot analysis using an antibody raised against phosphorylated cSrc. Maximal phosphorylation of cSrc was observed at 5 minutes (3.99 ± 0.8 fold above vehicle treated control, $P<0.05$, Figure 4.9), which is within the time frame of maximal ERK 1/2 phosphorylation (i.e. 5 - 10 minutes). These data demonstrate that PROK1 via PROKR1 induces phosphorylation of cSrc.

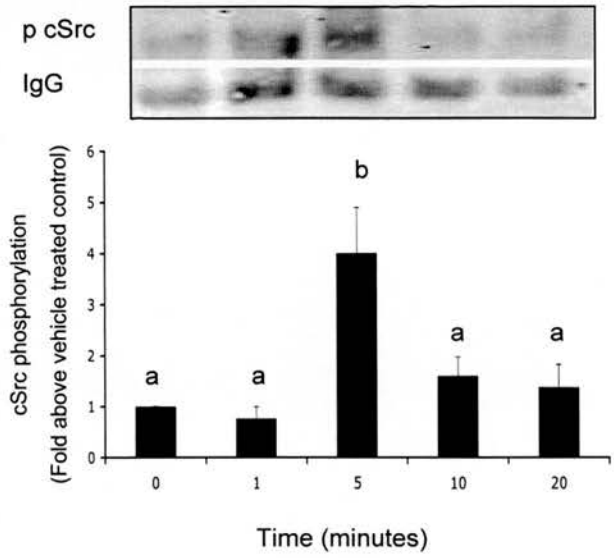


Figure 4.9. PROK1 induced cSrc phosphorylation in PROKR1 Ishikawa cells. PROKR1 Ishikawa cells were treated with 40nM PROK1 for 0, 1, 5, 10 and 20 minutes. 1mg of protein was immunoprecipitated using anti-phosphotyrosine (PY20) agarose preconjugate. Protein was resolved by SDS-PAGE followed by Western immunoblot analysis using specific antibodies against phosphorylated cSrc (p cSrc). The total amount of protein in cell lysates was determined by quantifying the IgG band (IgG). cSrc phosphorylation was normalised against IgG. Data are expressed as fold above vehicle treated control. A representative Western immunoblot is shown with semi-quantitative densitometric analysis. The data are presented as mean ± SEM. (b is significantly different to a, $P<0.05$).

4.3.3.5.PROK1 induces phosphorylation of the epidermal growth factor receptor.

Chemical inhibitors studies and dominant negative studies also suggested a role for phosphorylation of the EGFR receptor tyrosine kinase in the signalling cascade activated by PROK1. In order to investigate the temporal regulation of EGFR phosphorylation, PROKR1 Ishikawa cells were treated with 40nM PROK1 for 0, 1, 5, 10 and 20 minutes, lysed and

immunoprecipitated with anti phosphotyrosine (PY20) agarose conjugate in order to immunoprecipitate tyrosine phosphorylated EGFR. Immunoprecipitated proteins were subjected to Western immunoblot analysis using specific antibodies raised against phosphorylated EGFR. Maximal phosphorylation of EGFR occurred at 5 minutes (2.02 ± 0.27 fold above vehicle treated control, $P<0.01$, Figure 4.10). This phosphorylation was sustained up to 20 minutes following treatment with PROK1. Maximal phosphorylation of the EGFR occurred within the time frame of maximal ERK 1/2 phosphorylation. These data demonstrate that PROK1 via PROKR1 induces phosphorylation of EGFR.

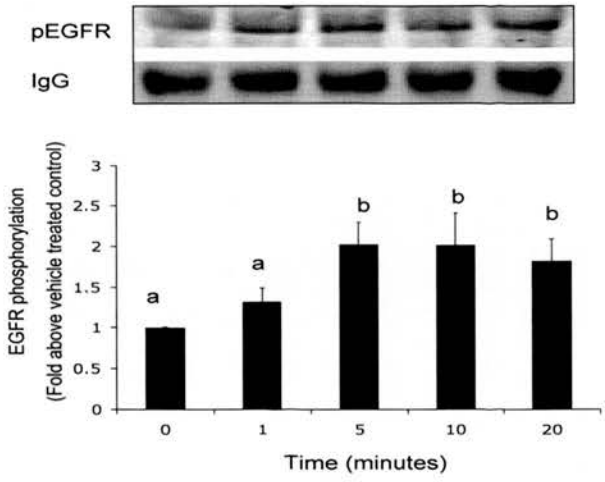


Figure 4.10. PROK1 induced EGFR phosphorylation in PROKR1 Ishikawa cells. PROKR1 Ishikawa cells were treated with 40nM PROK1 for 0, 1, 5, 10 and 20 minutes. 1mg protein was immunoprecipitated with anti phosphotyrosine (PY20) agarose conjugate. Protein was resolved by SDS-PAGE followed by Western immunoblot analysis using specific antibodies against phosphorylated EGFR (pEGFR). The total amount of protein in cell lysates was determined by quantifying the IgG band (IgG). EGFR phosphorylation was normalised against IgG. Data are expressed as fold above vehicle treated control. A representative Western immunoblot is shown with semi-quantitative densitometric analysis. The data are presented as mean ± SEM. (b is significantly different to a, $P<0.01$).

4.3.3.6.PROK1 induces phosphorylation of cSrc via Gq and Ca^{2+}

Phosphorylation of cSrc and EGFR is maximal at 5 minutes. However cSrc phosphorylation is transient and has diminished by 10 minutes while EGFR phosphorylation appears to be sustained until 20 minutes. It is suggested that intracellular cSrc can induce transactivation of the EGFR (Wetzker,

2003). In order to determine whether cSrc phosphorylation occur prior to EGFR and ERK 1/2 phosphorylation chemical inhibitors of Gq protein (YM254890), Ca^{2+} (BAPTA-AM), cSrc (PP2), EGFR (AG1478) and MEK (PD98059) were used. PROK1 induced cSrc phosphorylation (Figure 4.11, lane 2) was abolished by co-treatment with Gq inhibitor (Figure 4.11, lane 3), calcium inhibitor (Figure 4.11, lane 4) and specific cSrc inhibitor (Figure 4.11, lane 5). PROK1 induced cSrc phosphorylation was not affected by pre-treatment with inhibitors of EGFR (Figure 4.10, lane 6) or MEK (Figure 4.10, lane 7). These data suggest that in the signalling hierarchy cSrc is below activation of calcium signalling but above EGFR and ERK 1/2 phosphorylation.

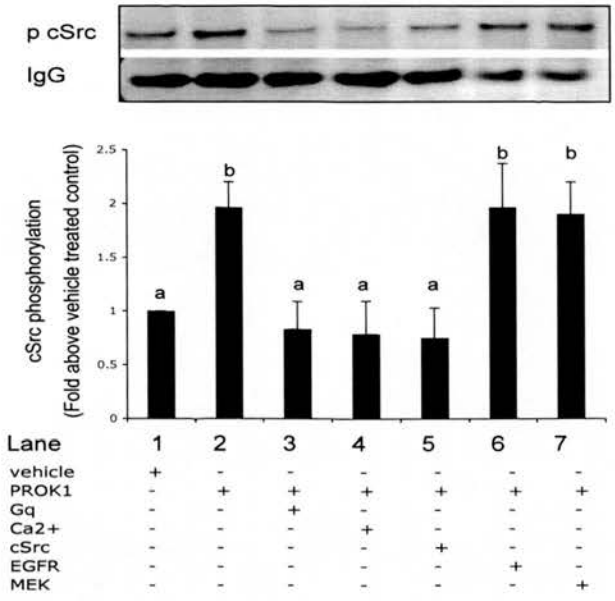


Figure 4.11. Chemical inhibitors of Gq protein, Ca^{2+} and cSrc but not EGFR or MEK inhibit cSrc phosphorylation. PROKR1 Ishikawa cells were pre-treated with inhibitors of Gq protein (YM254890), Ca^{2+} (BAPTA-AM), cSrc (PP2), EGFR (AG1478) and MEK (PD98059), prior to stimulation with 40nM PROK1 for 5 minutes. 1mg protein was Immunoprecipitated with anti-phosphotyrosine (PY20) agarose conjugate antibodies. Protein was resolved by SDS-PAGE followed by Western immunoblot analysis using specific antibodies against phosphorylated cSrc (p cSrc). The total amount of protein cell lysates was determined by quantifying the IgG band (IgG). cSrc phosphorylation was normalised against IgG. Data are expressed as fold above vehicle treated control. PROK1 mediated cSrc phosphorylation (lane 2) was inhibited by pre-treatment with inhibitors of Gq (lane 3), Ca^{2+} (lane 4) and cSrc (lane 5), but not inhibitors of EGFR (lane 6) or MEK (lane 7). + denotes presence of agent, - denotes absence of agent. A representative Western immunoblot is shown with semi-quantitative densitometric analysis. The data are presented as mean ± SEM. (b is significantly different to a $p<0.05$).

4.4. Discussion

This chapter describes the establishment of an endometrial epithelial cell line stably expressing PROKR1 and downstream protein phosphorylation cascade activation upon PROK1-PROKR1 interaction. One group describes prokineticin receptors as being Gi coupled (Lin R *et al*, 2002) while other groups describe them as being Gq coupled (Lin DC *et al*, 2002, Masuda *et al*, 2002, Soga *et al*, 2002). This may seem inconsistent, however it may be accounted for by the fact that the work describing Gi coupling was performed in primary cells while the work describing Gq coupling was performed in CHO (Chinese hamster ovary) and HEK293 (human embryonic kidney) cells transfected with prokineticin receptors. There is a precedent for this difference in signalling, presented by the example of GnRH receptors. Gq has been described as the predominant G-protein coupled to the GnRH receptor (Stojilkovic *et al*, 1995, Poulin *et al*, 1998, Hseih *et al*, 1992). However coupling to Gi has been demonstrated in uterine leiomyosarcomas (Imai *et al*, 1996), uterine endometrial carcinomas (Imai *et al*, 1997) and human prostate cancers (Limonta *et al*, 1999). Coupling of the GnRH receptor to Gs has also been demonstrated in rat pituitary cells (Janovick *et al*, 1993). These examples demonstrate that G-protein coupling may be different depending on the tissue examined, the pathological state of the tissue and, potentially, the species examined. This study demonstrates that in PROKR1 Ishikawa cells, PROKR1 is Gq coupled and activates inositol phosphate mobilisation. Whether this represents the *in vivo* situation requires clarification with studies in primary endometrium tissue.

The inositol phosphate mobilisation induced by PROK1 in PROKR1 Ishikawa cells was approximately 3-fold above vehicle treated controls. To ensure that only Gq was activated, the cAMP production and pertussis toxin sensitivity of PROK1 induced ERK 1/2 phosphorylation in PROKR1 Ishikawa cells was investigated. PROKR1 Ishikawa cells did not induce, or inhibit, production of cAMP in response to 40nM PROK1 at any time. However, forskolin did initiate cAMP production indicating the cells are responsive and capable of activating Gs to produce cAMP. Pre-treatment of PROKR1 Ishikawa cells with 200ng/ml pertussis toxin overnight or for one hour prior to treatment with 40nM PROK1 did not inhibit the PROK1

induced ERK 1/2 phosphorylation. Taken together, these data indicate that PROKR1 is not Gi or Gs coupled and activation of downstream signalling appears to occur solely via activation of Gq in PROKR1 Ishikawa cells.

Activation of inositol phosphate mobilisation can be considered as one of the first steps in activation of intracellular protein phosphorylation cascades (Belcheva & Coscia, 2002). Protein phosphorylation in PROKR1 Ishikawa cells was investigated by examining PROK1 induced phosphorylation of ERK 1/2. PROK1 has previously been demonstrated to induce phosphorylation of ERK 1/2 (Lin R *et al*, 2002, Lin DC *et al*, 2002). However, as PROK1 signalling had not been previously investigated in PROKR1 Ishikawa cells the concentration of PROK1 and time point at which maximal phosphorylation of ERK 1/2 would be induced was unknown. Treatment of PROKR1 Ishikawa cells with a range of concentration of PROK1 for 5 minutes indicated that 40nM PROK1 induced maximal phosphorylation of ERK 1/2. This is in line with previous studies where doses ranging from 10 - 40nM PROK1 have been used in order to initiate signalling in response to PROK1 (Lin DC *et al*, 2002, Lin R *et al*, 2002). Investigation of the time frame of maximal ERK 1/2 phosphorylation in response to 40nM revealed ERK 1/2 to be maximally phosphorylated at 5 - 10 minutes in PROKR1 Ishikawa cells. Again, this corresponds with previous reports investigating PROK1 induced ERK 1/2 phosphorylation where maximal phosphorylation occurred at approximately 10 minutes (Lin R *et al*, 2002).

The molecules involved in the intracellular protein phosphorylation cascade activated by PROK1 upstream of ERK 1/2 phosphorylation are currently unknown. The potential proteins involved in transmitting the phosphorylation signal to ERK 1/2 were investigated initially by use of chemical inhibitors. Pre-treatment of PROKR1 Ishikawa cells with chemical inhibitors of Gq protein, phospholipase C-β (PLC-β), Ca^{2+}, cSrc, EGFR and MEK inhibited the PROK1 induced ERK 1/2 phosphorylation at 5 minutes implicating a role for these molecules in signalling to ERK 1/2. The involvement of Gq, PLC-β and Ca^{2+} in signalling to ERK 1/2 could have been anticipated, as PROKR1 is Gq coupled and activates mobilisation of inositol phosphate, which occurs via activation of PLC-β (Lin DC *et al*, 2002). Previous studies have also demonstrated activation of

calcium mobilisation upon prokineticin - receptor interaction (Lin DC *et al*, 2002, Masuda *et al*, 2002). Involvement of the non-receptor tyrosine kinase cSrc and receptor tyrosine kinase EGFR has not previously been implicated in PROK1 induced signalling to ERK 1/2. However, there is an increasing body of evidence suggesting that activation of non-receptor tyrosine kinases (e.g. cSrc) and transactivation of receptor tyrosine kinases (e.g. EGFR) is important in transmitting the signal in GPCR signalling to ERK 1/2 (Werry *et al*, 2005, Daub *et al*, 1997, Shah *et al*, 2004, Eguchi *et al*, 2003).

The use of chemical inhibitors suggested the involvement of multiple signalling intermediates in PROK1 induced ERK 1/2 phosphorylation. In order to confirm the roles of MEK, cSrc and EGFR, and investigate the role of the small monomeric GTPase Ras in signalling to ERK 1/2, cDNA encoding dominant negative isoforms of these signalling molecules were utilised. Dominant negative isoforms of signalling molecules are mutant molecules and are designed to produce an inactive form of the parent signalling molecule. This inactive form is dominant in the cells and is over-expressed compared with the endogenous active molecule. The mutations can prevent phosphorylation of the protein via an inactive kinase domain, or in the case of the EGFR produce a mutant receptor that yields unproductive heterodimers at the cell surface thereby preventing downstream intracellular signalling (Kashles *et al*, 1991). Co-transfection of Myc-tagged ERK with dominant negative isoforms of cSrc, EGFR Ras or MEK significantly inhibited the PROK1 induced elevation in Myc-tagged ERK phosphorylation. These data confirm a role for cSrc, EGFR and MEK in signalling to ERK 1/2 and suggest, for the first time, a role for Ras.

The data generated using chemical inhibitors of signalling molecules and dominant negative mutants of signalling molecules suggested cSrc and EGFR to be involved in PROK1 mediated ERK 1/2 phosphorylation. The effect of PROK1 on phosphorylation of cSrc and EGFR was therefore investigated. Stimulation of PROKR1 Ishikawa cells with PROK1 revealed phosphorylation of both cSrc and EGFR to be maximal at 5 minutes. These data demonstrate phosphorylation of cSrc and EGFR by PROK1 and, by their temporal regulation, robustly suggest their role in signalling to ERK 1/2. Subsequently, the position of cSrc and EGFR in the

signalling hierarchy mediated by PROK1 was assessed. It is suggested that cSrc activation may control ectodomain shedding of HB-EGF required for transactivation of the EGFR (Wetzker *et al*, 2003). Phosphorylation of cSrc may therefore occur prior to EGFR phosphorylation. The data presented herein may support this assertion as cSrc phosphorylation peaks at 5 minutes and rapidly disappears whereas EGFR phosphorylation is sustained from 5 - 20 minutes. In order to determine if cSrc phosphorylation does indeed occur prior to EGFR phosphorylation, the effect of chemical inhibitors on cSrc phosphorylation was examined. Co-treatment of PROKR1 Ishikawa cells with PROK1 and inhibitors of Gq protein, Ca^{2+} and cSrc inhibited PROK1 induced cSrc phosphorylation. However inhibitors of EGR and MEK did not inhibit PROK1 induced cSrc phosphorylation, suggesting that cSrc is positioned upstream of EGFR and ERK 1/2 in the signalling hierarchy of PROK1 induced signalling, as has been demonstrated for other GPCR's (Daub *et al*, 1997, Daub *et al*, 1998, Luttrell *et al*, 1996).

This chapter describes the establishment of an endometrial cell line stably expressing functional PROKR1 and characterises signalling mediated by PROK1 via PROKR1. These data describe for the first time the mechanism of signalling induced by PROK1 in endometrial epithelial cells. This study demonstrates that PROKR1 couples to Gq but not Gs or Gi protein in PROKR1 expressing Ishikawa cells. The data also demonstrates sequential cSrc, EGFR and ERK 1/2 phosphorylation and the specific pathway that mediates ERK 1/2 phosphorylation via PLC-β - Ca^{2+} - cSrc - EGFR - MEK.

Chapter 5 - Target genes for PROK1 action

5.1. Introduction

In previous chapters the tissue localisation of the prokineticins and signalling induced by PROK1-PROKR1 was described. However, there is currently no data available on the downstream activation of gene expression induced by PROK1 in any cell models, tissues or pathological conditions and surprisingly little speculation upon the same. In an attempt to investigate activation of gene expression downstream of PROK1 induced protein phosphorylation, and to develop some idea of the function PROKR1-PROK1 may play in the endometrium, global gene array analysis was performed. As indicated in chapter 3, PROK1 expression is temporally regulated across the menstrual cycle and preferentially binds and activates PROKR1 (Lin DC *et al*, 2002). This therefore appears to be the most relevant system to study with reference to physiological function and prokineticin induced gene activation during the menstrual cycle.

The first step of this study was to perform a genome-wide gene expression scan to identify target genes for PROK1-PROKR1. Gene expression microarrays are currently the core technology for such purposes. Gene array analysis has been used in a large number of studies examining gene expression in the human endometrium across the menstrual cycle and after treatment with e.g. hCG or mifepristone in the endometrium of humans and other species such as baboon (Kao *et al*, 2002, Carson *et al*, 2002, Riesewijk *et al*, 2003, Borthwick *et al*, 2003, Mirkin *et al*, 2005, Talbi *et al*, 2006, Sherwin *et al*, 2007, Catalano *et al*, 2003). However, concerns are still raised with regards the reliability and consistency of microarray data analysis. There are a number of advantages and disadvantages associated with performing gene array experiments. Clearly, one of the major advantages is the ability to examine the expression of a large number of genes within a single RNA sample and has been used in diverse investigations, specifically within the field of endometrial research to compare expression of genes within the endometrium taken during different stages of the menstrual cycle or treated with contraceptives as indicated above. Another advantage of of such microarray technology lies in the fact that many of the processes are fully automated, reducing the possibility of technical and operator error. Assuming the starting material is of high quality and the

microarray chips are suitable (important as the chips have a limited shelf life) then assays performed at the same time should be highly comparable,

A major disadvantage of microarray studies arises when estimating the number of samples required giving statistically meaningful results, Application of power calculations can aid in this decision. Power calculations can be applied to predict the appropriate sample size required to achieve adequate power i.e. the probability that the null hypothesis will be rejected. In this study the null hypothesis was that PROK1 would not regulate expression of genes in PROKR1 Ishikawa cells when compared to vehicle treated control samples. In samples taken from in vivo studies the power calculations usually indicate that a large sample number is required to produce statistically significant data. However, in a homogenous cell line variation should be low, therefore a smaller sample size may be used. False positive results can also be a disadvantage of microarray experiments depending on the exclusion criteria set. In this study the criteria of fold changes of ≥1.5 fold and application of statistical criteria to control for the false discovery rate in multiple testing according to the Benjamini and Hochberg method (1995) should protect against the inclusion of any false positive data. However, the gene array results were not taken as absolute values and a number of genes were chosen for validation experiments at 8 hours in order to ensure the validity of the gene array data.

Mistakes made at the discovery stage may lead to a waste of resources in validation experiments performed after the gene array analyses. In order to obtain high confidence results it was decided to perform a cross-platform study using two different commercial platforms, the Affymetrix GeneChip® Human Genome U133 plus 2.0 and the AB1700 v.2 Applied Biosystems Human Genome Survey Microarrays. These platforms were selected as representing diverse approaches to array/probe designs, detection chemistries as well as gene contents/annotations (Design and Performance of the GeneChip® Human Genome U133 Plus 2.0 and Human Genome U133A 2.0 Arrays, Technical note, Affymetrix, 2003; White Paper: The Design and Annotation of the Human Genome Survey Microarray, ABI, 2004). Genes displaying concordance between the two different gene array platforms would increase confidence in the gene array

results and provide a greater degree of reliability when choosing genes to investigate further.

This study was therefore designed to examine the target genes regulated by PROK1 upon activation of PROKR1 in PROKR1 Ishikawa cells. This was investigated by gene array analysis on the two different array platforms. This dual platform approach provides a high degree of confidence in the concordant genes for further studies. Examination of gene expression regulated by PROK1 in the PROKR1 Ishikawa cells should enable potential functions of PROK1 in the endometrium to be delineated by comparisons with studies, which have previously demonstrated functionality of these genes.

5.2. Materials and methods

5.2.1. Cell Culture and PROK1 treatments

PROKR1 and WT Ishikawa cells were routinely maintained in DMEM F-12 Glutamax culture medium with 10% FCS and 1% antibiotics (500U/ml penicillin and 500µg/ml streptomycin) at 37°C and 5% CO_2. In addition PROKR1 Ishikawa cells were maintained in media containing 200µg/ml G418 antibiotic.

In order to prepare samples for analysis by gene array, PROKR1 Ishikawa cells were seeded out to a density of 5 x 10^5 cells in 5 x 6cm dishes for 40nM PROK1 treated cells and 5 x 6cm dishes for vehicle treated cells. On the day prior to stimulation, cells were incubated in serum free medium for at least 16 hours. The following day 40nM PROK1 or vehicle was added to each of 5 dishes of cells for 8 hours. The cells were then washed in PBS and RNA extracted as indicated below. After RNA extraction the 5 samples from one experiment, if they passed the inclusion criteria indicated in Section 5.2.2, were pooled. This was performed to reduce the risk of single sample variation. This was repeated 3 times in total to yield 3 different samples (which represented n=15 in total, given the low variability in homogenous cell lines it was considered that this would represent an adequate sample size to

provide statistically meaningful results) that were treated with 40nM PROK1 or vehicle.

5.2.2. RNA extraction and quantification

RNA was extracted from PROKR1 Ishikawa cells treated with 40nM PROK1 or vehicle for 8 hours using the RNeasy RNA extraction kit supplied by Qiagen. Briefly, RNeasy lysis buffer (containing β-mercaptoethanol) was added to the cells after washing with PBS. Cells were harvested by scraping with a rubber policeman (cell scraper), transferred to RNase free eppendorf tubes and homogenised by passing the lysate through a 0.22 bore needle a number of times. Cell lysates were then stored at -80°C before processing for RNA extraction following the manufacturers recommendations. Briefly the lysates were applied to silica columns, which capture the RNA, allowing it to be washed and DNase treated before elution of the RNA from the column with RNase free water. RNA samples were initially quantified using the Genequant spectrophotometer; this gave an indication of their concentration. The RNA was then diluted to allow quantification and assessment the quality of the samples on the 2100 Bioanalyser and RNA nanochip system (Agilent Technologies, Stockport). Certain criteria were set for the inclusion of RNA samples within the RNA pool. The criterion for sample inclusion in the gene array was A260/A280> 1.9; this was assessed by the genequant spectrophotometer. The quality was further assessed with the RNA nanochip system; this gave an RIN (RNA integrity number), which should be around 2, and a value for 18s/28s, which should also be around 2. Only if the samples passed these quality control tests were they included in the sample RNA pool. The 5 plates from each treatment were therefore pooled to create one representative RNA sample. The RNA was prepared and hybridized on 6 AB1700 (n=3 40nM PROK1 treated and n=3 vehicle treated) and 6 Affymetrix (n=3 40nM PROK1 treated and n=3 vehicle treated) arrays respectively. Hybridisation of the RNA samples to the gene arrays was performed commercially by Geneservice.

5.2.3. cRNA preparation and hybridisation to gene chips

5.2.3.1.ABI

The cRNA is produced by reverse transcriptase in vitro transcription (RT-IVT). Initially the reverse transcriptase enzyme incorporates deoxynucleotide in the synthesis of single stranded c DNA from sample RNA. The use of T7 poly dT primer adds the T7 polymerase promoter to the 5′ end of the single stranded c DNA transcript. This is followed by 2nd strand synthesis. RNase H degrades the RNA in DNA-RNA duplexes to provide priming sites for DNA polymerase to synthesise the 2nd strand DNA. The c DNA is subsequently purified; this washes away salts buffers, unincorporated nucleotides, primers and degraded RNA. The double stranded c DNA is then eluted in a solution suitable for the IVT reaction. During the IVT labelling the T7 polymerase incorporates ribonucleotides and digoxigenin (DIG)-UTP to synthesise copy RNA (cRNA) from double stranded c DNA containing the T7 promoter. This step results in 100- to 1000-fold amplification of targets. The c RNA is subsequently purified, this washes away salts, buffers etc, as indicated above. The cRNA is then eluted in a solution suitable for hybridisation on c RNA to the 1700 microarray.

Prior to chemiluminescent detection the cRNA must be fragmented and hybridised to the microarray. Divalent cations catalyse the 2′hydroxyl cleavage of RNA. This c RNA fragmentation reduces the secondary structure and improves hybridisation kinetics. Prior to hybridisation the microarrays are incubated with blocking agents to prevent non-specific hybridisation, the DIG labelled cRNA is then hybridised to probes on the microarray. Washes reduce non-specific hybridisation of the cRNA to the microarray and prepare the chip for antibody binding. In preparation for application of antibody, blocking buffer is again added to the microarray to prevent non-specific antibody binding. Anti-digoxigenin alkaline phosphatase labelled antibody is added to bind digoxigenin in the cRNA targets, the RT and IVT controls and the chemiluminescent control probes on the microarray. Washing steps again remove unbound antibody and prepare the microarray for the chemiluminescent reaction. An enhancing solution

is added to the microarray, this acts to increase the quantum yield of the chemiluminescent reaction, the chemiluminescent substrate is added which reacts with the alkaline phosphatase label, resulting in dephosphorylation of the substrate, this reaction emits a light signal. In order to detect this light emitting reaction, the microarray is loaded onto a heated stage of the applied biosystems 1700 chemiluminescent microarray analyzer, the analysis focuses on the microarray and obtains the chemiluminescent and fluorescent images of the microarray.

Clearly, in order to control for technical variation, differences in labelling, hybridisation, antibody binding and chemiluminescent detection a number of controls are required, these are absolutely essential in the final analysis of the microarray. These controls include blank features, control ladders, hybridisation controls, internal control probe and target, IVT controls, negative controls, RT controls and spatial normalisation controls. These parameters are all utilised in the generation of a quality check report which is required in the microarray analysis.

The signal intensity emitted at the blank features provides information about the background signal for every region of the microarray and cross talk from other regions. Non-specific binding of cRNA, antibodies, alkaline phosphatase or other chemical/mechanical processes may result in background signal. Signals emitted from the blank features indicate the level of non-specific signal generated from the assay. The software calculates the amount of background chemiluminescent signal from blank features and uses the correction to compare all other chemiluminescent signals on the microarray.

Control ladders are used to perform quality checks of assay spotting and chemistry, compare reproducibility of spotting across array batches and quality check the chemiluminescent detection chemistry. They also demonstrate the sensitivity and dynamic range of chemiluminescent detection chemistry. Fiducial control ladders produce signals which are independent of labelling and

hybridisation, they therefore indicate that the chemiluminescent reaction was successful. Signal intensity variability indicates differences in spotting and attachment efficiencies across various batches of assays.

The hybridisation controls are used to indicate successful hybridisation and stringency and can also be used as a spatial normalisation control. Hybridisation controls consist of 3 DIG labelled oligo control targets and 3 unlabelled probes which are spotted onto the microarray. The DIG labelled oligo targets are added to the microarray with the DIG labelled cRNA targets. The hybridisation controls on the microarray are designed to hybridise with the DIG-labelled oligo targets. The presence of a signal in these targets indicates that the hybridisation has occurred successfully and the strength of the signal indicates the stringency of this hybridisation.

The internal control probe and target are used to normalise the chemiluminescent signal. The IC target is added to the hybridisation mixture and during the hybridisation reaction this hybridises to every IC probe which is co-spotted at every feature in the microarray.

The IVT controls demonstrate the IVT labelling reaction has taken place and the efficiency of the reaction. Negative controls demonstrate the level of the non-specific background signals from all probes on the microarray. Non-specific binding can result from cross hybridisation, binding of AP enzyme or other chemical processes that lead to background chemiluminescent signal. The software calculates the amount of background chemiluminescent signal and corrects for this when measuring all other chemiluminescent signals on the microarray. The RT controls indicate, again, if and how well the reaction has worked.

5.2.3.2. Affymetrix

Preparation and hybridisation was described in main materials and methods (Section 2.9). Briefly, cDNA synthesis and Biotin labelling of cRNA were carried

out using the Ambion Message Amp II cRNA kit. Samples were fragmented, prepared for hybridisation using the Affymetrix hybridisation control kit and hybridised to GeneChip® Human Genome U133 plus 2.0 arrays. The GeneChip arrays were subsequently stained, washed on the fluidics station and scanned.

5.2.4. Data analysis

This analysis was performed by Geneservice. The ABI software uses spatial normalisation control signal to remove any schematic spatial trends in the feature ratios across the array. These potential spatial differences across the array might be caused by non-uniform LED fluorescence illumination, non-uniform hybridisation, or non-uniform focus of the microarray. The spatial normalisation controls are distributed throughout the microarray and should produce the same CL/FL signal ratios.

After the ICP normalisation, the algorithm identifies the spatial trends for CL/FL signal rations and derives correction factors by interpolating between the spatial normalisation control CL/FL ratios. The algorithm then removes the spatial trends from all feature ratios using local correction factors from the nearest normalisation control. These controls should avoid bias in labelling of samples. Finally, using these control data a quality check report is generated. As indicated above, this is essential in the final analysis of the microarray. The performance from the QC report indicates the sample signal information, the background signal, the median signal to noise (S/N) values and the number of genes detected above a set threshold. The S/N threshold in this analysis is set at >3 for inclusion in the dataset. The software automatically generates flags for poor or missing spots, if the flag measurement is >100 in this analysis the data were removed from the dataset.

The data, after subjection to these pre-processing steps, was normalised using variance stabilising normalisation (vsn). This is a statistical model used for microarray expression data. This model comprises data calibration, quantification of differential expression and quantification of measurement error. Application of variance stabilising transformation to achieve a constant signal: noise ration for

intensity measurements results in a difference statistic which displays approximately variance independent of the spot intensity, i.e. variance is approximately constant along the whole intensity range. This transformation also accounts for calibration, covering different behaviour of dyes or variations between samples and assays. It is important that intensity measurements must be on a common scale before they can be compared. The estimation of the calibration parameters is simplified through application of vsn and the difference statistic is obtained as the difference between the transformed data of the individual samples, allowing comparison of two samples and therefore calculation of fold change. (Huber, 2002)

LIMMA is a software package used for the analysis of gene expression microarray data, especially the use of linear models for analysing experiments and the assessment of differential expression. The differential expression methods apply to all assay platforms and treat them in a unified way. LIMMA analysis allows the fitting of a linear model, smoothing of the standard errors by application of a Bayes empirical method to a common value. With one channel microarray data, linear modelling is much the same as ordinary ANOVA or multiple regression except that a model is fitted for every gene. LIMMA provides functions which summarise the results of the linear model, perform hypothesis tests and adjust the p-values for multiple testing. Results provided by LIMMA include (log) fold changes, standard errors, t-statistics and p-values. The moderated t-value is the basic statistic which is used for significance analysis, this is computed from each probe. The moderated t-statistic has the same interpretation as an ordinary t-statistic except that the standard errors have been moderated across genes, i.e. shrunk towards a common value. This is performed using a Bayesian model and enables the borrowing of information from the ensemble of genes to aid with the inference about each individual gene. Moderated t-statistics result in p-values in the same way as ordinary t-statistics with the exception that the degrees of freedom are increased; this reflects the greater reliability associated with the smoothed standard errors. The common p-value is the associated p-value which results after adjustment for multiple testing. In this case the p-values were adjusted for multiple testing to control for the false discovery rate with the Benjamini and Hochberg method (1995).

The false discovery rate control indicates that if all genes with p-values below threshold $p>0.05$ are selected as differentially expressed then the expected proportion of false discoveries in the selected group is controlled to be less than the threshold value, in this case 5%.

Cross mapping between AB1700 probes and Affymetrix probesets was courtesy provided by Applied Biosystems. All unmapped poorly annotated genes were removed from the analysis. All mapped genes yielding fold changes of more than 1.5 when PROK1 treated samples were compared with vehicle treated samples on both platforms were selected for further consideration. Genes were further filtered by their adjusted p-values with significance set at $p<0.05$.

Gene ontologies (GO) describe how gene products behave in a cellular context i.e. describe gene products in terms of their associated biological processes, cellular components and molecular functions. GO's were assigned to PROK1 regulated genes for biological processes using tools provided by the in the gene ontology database (www.geneontology.org).

Analysis of the expressed sequence tag tissue library database for PROK1 regulated genes were conducted using WebGestalt (Web based gene set analysis toolkit) in order to examine the tissue expression of PROK1 regulated genes.

5.2.5. PCR verification of gene expression

5.2.5.1.RNA extraction

In order to examine the temporal regulation of PROK1 target genes, PROKR1 and WT Ishikawa cells were treated with 40nM PROK1 or vehicle for 0, 2, 4, 6, 8, 12 and 24 hours. Media was removed and TRI reagent added to the plates for RNA extraction. TRI reagent cell lysates were transferred to phase-lock tubes and RNA extracted by the phenol-chloroform method of RNA extraction as described in Section 2.5. RNA samples were quantified using the genequant or nanodrop spectrophotometers.

5.2.5.2.Taqman PCR analysis

Expression of mRNA within cultured cells was examined by Taqman PCR in order to quantify expression and detect low expression transcripts as described in Section 2.5. Briefly, cDNA was prepared in a random hexamer primed reaction using 200ng of RNA per reaction.

Quantitative PCR was conducted on the cDNA using specific primer-probe mixtures to detect COX-2, LIF, IL6, IL-8 or IL-11 (Sequences given in Table 5). The Probe was given a FAM fluorescent label. Gene expression was normalised by including Vic labelled primer-probe mix to detect the 18s ribosomal subunit (Sequences given in Table 5) as a loading control for the amount of cDNA added in each sample.

Reaction mixtures were loaded, in duplicate onto a 96-well MicroAmp fast optical reaction plate (Applied Biosystems) for analysis on an ABI 7900 HT Fast Real-Time PCR machine (Applied Biosystems). Data were analysed and processed using Sequence detector version 1.6.3 (Applied Biosystems) according to the manufacturers instructions. Results were expressed as relative to a positive RNA standard included in all reactions. The data was analysed using the comparative C_T method for relative quantification.

5.2.6. Statistics

Other than where indicated, statistical analysis of the data in this study was carried out by ANOVA with Fishers PLSD (predicted least squares difference) applied, using Statview 5.0 (Abacus Concepts). The data is presented as mean ± SEM of at least 3 experiments.

5.3. Results

5.3.1. Gene expression altered upon treatment of PROKR1 Ishikawa cells with PROK1

Genes were ranked if they were changed (up-regulated or down-regulated) by greater than 1.5 fold on both platforms. Multiples, i.e. probes representing the same gene and unclassified genes were removed and a median value assigned. This revealed regulation of 277 genes on both arrays of which 226 were up-regulated while 51 were down-regulated (Table 11). Concordance in changes in gene expression between the two gene array platforms was examined with application of adjusted p-values of $p<0.05$. Application of these further selection criteria reduced the list to 49 genes, with 46 genes up-regulated and 3 genes down regulated (Table 12).

5.3.2. Classification of PROK1 regulated genes into gene ontologies

In order to determine functional processes which may be regulated by PROK1 *in vivo*, the genes induced by PROK1 in the PROKR1 Ishikawa cells were classified into gene ontology (GO) groups for biological processes. GO's describe how gene products behave in a cellular context and gene products are assigned GO terms, which define a biological process, cellular component or molecular function. Analysis for significantly over-represented Gene Ontology (GO) annotations within the gene list revealed 3 significant themes; cellular morphogenesis, cell motility/chemotaxis and blood vessel development/morphogenesis (Table 13). Analysis of the expressed sequence tag (EST) tissue library database (Zhang B *et al*, 2005) indicated that genes regulated by PROK1 are significantly over-expressed in uterine tissue compared to other tissue libraries, indicating that although the cell line is derived from cancer tissue and studies performed *in vitro*, the response is likely to be representative of uterine cells.

Table 11. Treatment of PROKR1 Ishikawa cells with 40nM PROK1 for 8 hours revealed 277 genes to be regulated with fold changes (expressed relative to vehicle treated PROKR1 cells) of greater than 1.5 on each gene array. Analysis revealed 226 genes to be up-regulated by greater than 1.5 fold on both arrays while 51 genes were down-regulated by greater than 1.5 on both arrays, Values represent median fold changes of gene expression on ABI 1700 and Affymetrix gene array platforms following treatment with 40nM PROK1. Positive denotes up-regulation (numbers in red); negative denotes down-regulation (numbers in blue).

Gene Symbol	Gene Product	ABI	Affy	positive	negative
ABHD7	abhydrolase domain containing 7	1.5	1.7	1.6	
ACATE2	Mitochondrial Acyl-CoA Thioesterase	1.7	1.6	1.6	
ACSL4	acyl-CoA synthetase long-chain family member 4 isoform 2	1.5	1.6	1.5	
ACTA2	alpha 2 actin	1.8	1.7	1.7	
ACTN1	actinin, alpha 1	2.0	1.6	1.8	
ADM	adrenomedullin	1.7	1.5	1.6	
AKAP12	A-kinase anchor protein 12 isoform 2;A-kinase anchor protein 12 isoform 1	3.7	1.9	2.8	
ALDH1A1	aldehyde dehydrogenase 1A1	0.2	0.3		3.9
ANKRD1	cardiac ankyrin repeat protein	2.8	2.5	2.6	
AREG	amphiregulin preproprotein	6.1	4.4	5.3	
ARGBP2	Arg/Abl-interacting protein 2 isoform 2;Arg/Abl-interacting protein 2 isoform 1	0.6	0.5		1.7
ARHI	ras homolog I	1.8	1.6	1.7	
ATF3	activating transcription factor 3 delta Zip isoform;activating transcription factor 3 long isoform	4.5	3.2	3.9	
BACH2	BTB and CNC homology 1, basic leucine zipper transcription factor 2	2.3	1.7	2.0	
BCAR3	breast cancer antiestrogen resistance 3	2.5	2.0	2.3	
BCL10	B-cell CLL/lymphoma 10	1.5	1.7	1.6	
BCL6	B-cell lymphoma 6 protein	0.5	0.6		1.9
BHLHB2	differentiated embryo chondrocyte expressed gene 1	2.4	2.2	2.3	
BNC1	basonuclin 1	2.7	2.1	2.4	
C10orf48	chromosome 10 open reading frame 48	1.5	1.7	1.6	
C11orf8	chromosome 11 open reading frame 8	0.3	0.4		2.7
C18orf25	chromosome 18 open reading frame 25	1.7	2.0	1.9	

C1orf106	chromosome 1 open reading frame 106	2.0	1.5	1.8	
C1orf168	chromosome 1 open reading frame 168	0.4	0.5		2.2
C1orf24	chromosome 1 open reading frame 24	2.7	1.6	2.1	
C2orf26	chromosome 2 open reading frame 26	2.5	2.5	2.5	
C3orf6	chromosome 3 open reading frame 6 long isoform;chromosome 3 open reading frame 6	1.9	1.9	1.9	
CALD1	caldesmon 1 isoform 5;caldesmon 1 isoform 4;caldesmon 1 isoform 3;caldesmon 1 isoform 2;caldesmon 1 isoform 1	2.0	1.8	1.9	
CAPN2	calpain 2, large subunit	2.0	1.9	2.0	
CCL20	chemokine (C-C motif) ligand 20	12.0	2.8	7.4	
CCNA1	cyclin A1	2.2	1.9	2.0	
CCNB2	cyclin B2	0.6	0.6		1.7
CD24	CD24 antigen	0.6	0.6		1.6
CD44	CD44 antigen isoform precursor	2.0	1.8	1.9	
CDC20	cell division cycle 20	0.5	0.6		1.7
CDCA3	trigger of mitotic entry 1	0.6	0.7		1.6
CDCP1	CUB domain-containing protein 1 isoform 2;CUB domain-containing protein 1 isoform 1	2.2	1.8	2.0	
CDH2	cadherin 2, type 1 preproprotein	2.4	1.8	2.1	
CDKN1A	cyclin-dependent kinase inhibitor 1A	2.8	1.8	2.3	
CDKN2C	cyclin-dependent kinase inhibitor 2C	0.6	0.6		1.7
CENTD1	centaurin delta 1 isoform b;centaurin delta 1 isoform a	1.6	1.7	1.7	
CITED2	Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2	1.9	1.6	1.7	
CLDN1	claudin 1	1.5	1.6	1.5	
COL4A2	alpha 2 type IV collagen preproprotein	2.0	2.0	2.0	
COL5A2	alpha 2 type V collagen preproprotein	0.6	0.5		1.8
COL8A1	alpha 1 type VIII collagen precursor	2.1	2.0	2.0	
CORO1C	coronin, actin binding protein, 1C	2.5	2.0	2.3	
CRABP1	cellular retinoic acid binding protein 1	0.6	0.7		1.5
CRYAB	crystallin, alpha B	3.8	2.0	2.9	
CSRP1	cysteine and glycine-rich protein 1	1.8	1.5	1.7	
CTGF	connective tissue growth factor	5.4	4.6	5.0	
CUGBP2	CUG triplet repeat, RNA binding protein 2	1.9	1.5	1.7	

CXCL2	chemokine (C-X-C motif) ligand 2	3.6	1.7	2.7	
CXCL3	chemokine (C-X-C motif) ligand 3	5.2	2.1	3.6	
CXCR4	chemokine (C-X-C motif) receptor 4	2.0	1.6	1.8	
CXXC5	CXXC finger 5	0.4	0.5		2.2
CYR61	cysteine-rich, angiogenic inducer, 61	3.6	2.2	2.9	
DAF	decay accelerating factor for complement (CD55, Cromer blood group system)	21.8	11.9	16.8	
DAPK3	death-associated protein kinase 3	1.5	1.9	1.7	
DAPP1	dual adaptor of phosphotyrosine and 3-phosphoinositides	3.5	1.6	2.5	
DCAMKL1	doublecortin and CaM kinase-like 1	2.9	2.2	2.6	
DCBLD2	discoidin, CUB and LCCL domain containing 2	2.4	1.8	2.1	
DHRS2	dehydrogenase/reductase (SDR family) member 2 isoform 2;dehydrogenase/reductase (SDR family) member 2 isoform 1	0.6	0.6		1.7
DIAPH3	diaphanous homolog 3 (Drosophila)	1.7	1.5	1.6	
DKFZp451A211	DKFZp451A211 protein	3.1	1.5	2.3	
DKK1	dickkopf homolog 1	10.6	6.4	8.5	
DLG7	discs large homolog 7	0.6	0.6		1.7
DMBT1	deleted in malignant brain tumors 1 isoform precursor	6.1	1.8	4.0	
DNAJB2	DnaJ (Hsp40) homolog, subfamily B, member 2	1.8	1.6	1.7	
DNAJB9	DnaJ (Hsp40) homolog, subfamily B, member 9	2.0	1.7	1.8	
DSCR1	calcipressin 1 isoform c;calcipressin 1 isoform b;calcipressin 1 isoform a	5.7	4.7	5.2	
DTR	diphtheria toxin receptor (heparin-binding epidermal growth factor-like growth factor)	4.6	2.6	3.6	
DUSP1	dual specificity phosphatase 1	5.0	3.7	4.4	
DUSP14	dual specificity phosphatase 14	2.1	1.8	1.9	
DUSP3	dual specificity phosphatase 3	1.6	1.6	1.6	
DUSP4	dual specificity phosphatase 4 isoform 2;dual specificity phosphatase 4 isoform 1	13.3	9.3	11.3	
DUSP5	dual specificity phosphatase 5	8.6	5.9	7.2	
DUSP6	dual specificity phosphatase 6 isoform b;dual specificity phosphatase 6 isoform a	1.7	1.6	1.7	
EDN2	endothelin 2	7.7	3.2	5.4	
EGR1	early growth response 1	2.7	2.0	2.3	
EHD4	EH-domain containing 4	1.6	1.5	1.6	
EIF2AK3	eukaryotic translation initiation factor 2-alpha kinase 3	1.8	1.7	1.7	

EMP1	epithelial membrane protein 1	2.1	1.9	2.0	
EMX2	empty spiracles homolog 2	0.6	0.6		1.6
ENAH	enabled homolog	1.9	1.5	1.7	
EPHA2	ephrin receptor EphA2	1.9	1.8	1.8	
ERO1LB	endoplasmic reticulum oxidoreductin 1-Lbeta	1.6	1.5	1.6	
EXT1	exostosin 1	1.8	1.6	1.7	
F3	coagulation factor III precursor	7.2	4.2	5.7	
FER1L3	myoferlin isoform b;myoferlin isoform a	1.6	1.5	1.6	
FGD6	FYVE, RhoGEF and PH domain containing 6	2.0	1.6	1.8	
FGF1	fibroblast growth factor 1 (acidic) isoform precursor	1.7	1.7	1.7	
FGF2	fibroblast growth factor 2	1.8	1.8	1.8	
FGFR2	fibroblast growth factor receptor 2 isoform precursor	0.4	0.7		1.9
FHL2	four and a half LIM domains 2	3.8	2.8	3.3	
FLJ10156	hypothetical protein FLJ10156	0.6	0.7		1.5
FLJ22833	hypothetical protein FLJ22833	2.1	1.7	1.9	
FLJ39370	hypothetical protein FLJ39370	2.0	2.0	2.0	
FLRT3	fibronectin leucine rich transmembrane protein 3 precursor	0.5	0.6		1.9
FOSL1	FOS-like antigen 1	2.6	2.1	2.3	
FST	follistatin isoform FST344 precursor;follistatin isoform FST317 precursor	4.4	2.9	3.6	
G0S2	putative lymphocyte G0/G1 switch gene	0.5	0.6		1.8
G1P2	interferon, alpha-inducible protein (clone IFI-15K)	2.1	1.6	1.9	
GADD45A	growth arrest and DNA-damage-inducible, alpha	1.5	1.6	1.6	
GBE1	glucan (1,4-alpha-), branching enzyme 1 (glycogen branching enzyme)	2.5	2.2	2.3	
GEM	GTP-binding mitogen-induced T-cell protein	7.1	3.6	5.3	
GJB6	gap junction protein, beta 6 (connexin 30)	1.6	1.5	1.6	
GLIPR1	glioma pathogenesis-related protein	5.0	2.7	3.9	
GREM1	cysteine knot superfamily 1, BMP antagonist 1	16.2	8.5	12.4	
GSTM3	glutathione S-transferase M3	0.4	0.6		2.1
H2BFS	H2B histone family, member S	0.7	0.6		1.5
HERC4	hect domain and RLD 4	1.9	1.9	1.9	

HERPUD1	homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1	2.8	2.4	2.6	
HMGB2	high-mobility group box 2	0.6	0.6		1.6
HSPA5	heat shock 70kDa protein 5 (glucose-regulated protein, 78kDa)	2.1	1.9	2.0	
HSPB8	heat shock 27kDa protein 8	5.2	4.1	4.7	
ID3	inhibitor of DNA binding 3	2.0	1.5	1.8	
IER2	immediate early response 2	1.7	1.7	1.7	
IER3	immediate early response 3 isoform long;immediate early response 3 isoform short	11.5	7.0	9.2	
IFRD1	interferon-related developmental regulator 1	1.8	1.9	1.8	
IL11	interleukin 11 precursor	18.2	21.4	19.8	
IL18	interleukin 18 proprotein	2.4	1.9	2.1	
IL1RAP	interleukin 1 receptor accessory protein isoform 2;interleukin 1 receptor accessory protein isoform 1	2.3	1.7	2.0	
IL6	interleukin 6 (interferon, beta 2)	26.3	8.0	17.2	
IL8	interleukin 8 precursor	9.1	10.4	9.7	
INPP1	inositol polyphosphate-1-phosphatase	1.9	1.5	1.7	
INSIG1	insulin induced gene 1 isoform 2;insulin induced gene 1 isoform 3;insulin induced gene 1 isoform 1	2.7	2.7	2.7	
ITGB8	integrin, beta 8	1.9	2.0	2.0	
JAG1	jagged 1 precursor	1.6	1.5	1.6	
KIAA0063	KIAA0063 gene product	1.5	1.5	1.5	
KIAA0226	KIAA0226	1.8	1.6	1.7	
KIAA1295	SH3 and PX domains 2B	2.0	1.5	1.7	
KIAA1363	KIAA1363 protein	2.0	2.0	2.0	
KIF20A	kinesin family member 20A	0.4	0.5		2.2
KIT	v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog precursor	0.2	0.3		4.4
KLF3	Kruppel-like factor 3 (basic)	1.5	1.6	1.5	
KLF5	Kruppel-like factor 5	1.9	2.0	2.0	
KLHL21	kelch-like 21 (Drosophila)	1.9	1.7	1.8	
KRT17	keratin 17	12.4	5.2	8.8	
KRTAP2-2	keratin associated protein 2-2	2.0	4.0	3.0	
KRTAP2-4	keratin associated protein 2-4	6.5	4.0	5.3	
KRTHA4	type I hair keratin 4	9.5	2.1	5.8	

LAMA3	laminin alpha 3 subunit isoform 2;laminin alpha 3 subunit isoform 1	2.0	2.1	2.0	
LBH	hypothetical protein DKFZp566J091	8.7	5.7	7.2	
LCN2	lipocalin 2 (oncogene 24p3)	4.4	1.7	3.1	
LCP1	L-plastin	1.5	1.6	1.6	
LDLR	low density lipoprotein receptor precursor	2.5	2.2	2.4	
LGALS1	beta-galactosidase binding lectin precursor	2.1	1.6	1.8	
LGALS8	galectin 8 isoform b;galectin 8 isoform a	1.8	1.5	1.6	
LIF	leukemia inhibitory factor (cholinergic differentiation factor)	3.4	1.8	2.6	
LIPG	endothelial lipase precursor	1.8	1.6	1.7	
LMCD1	LIM and cysteine-rich domains 1	2.2	1.7	2.0	
LMNB1	lamin B1	0.5	0.6		1.8
LOC151963	similar to BcDNA:GH11415 gene product	2.2	1.9	2.0	
LOC54103	hypothetical protein LOC54103	2.4	1.8	2.1	
LOC83690	CocoaCrisp	2.2	1.9	2.1	
Lrp2bp	low density lipoprotein receptor-related protein binding protein	2.1	1.5	1.8	
LRRFIP1	leucine rich repeat (in FLII) interacting protein 1	1.9	1.9	1.9	
LUM	lumican	2.9	2.1	2.5	
MAFK	v-maf musculoaponeurotic fibrosarcoma oncogene homolog K	1.5	2.3	1.9	
MAIL	molecule possessing ankyrin repeats induced by lipopolysaccharide	4.2	2.2	3.2	
MAL	T-lymphocyte maturation-associated protein	0.4	0.4		2.6
MAMDC2	MAM domain containing 2	2.0	2.2	2.1	
MAP1B	microtubule-associated protein 1B isoform 2;microtubule-associated protein 1B isoform 1	1.8	2.1	1.9	
MAP3K4	mitogen-activated protein kinase kinase kinase 4 isoform b;mitogen-activated protein kinase kinase kinase 4 isoform a	1.9	1.6	1.8	
MGC11324	hypothetical protein MGC11324	3.8	2.2	3.0	
MGC14376	hypothetical protein MGC14376	1.8	1.6	1.7	
MGC26963	hypothetical protein MGC26963	1.6	1.8	1.7	
MGC29643	hypothetical protein MGC29643	0.5	0.5		2.1
MGC4677	hypothetical protein MGC4677	2.2	1.5	1.9	
MGLL	monoglyceride lipase	2.3	1.8	2.0	
MLP	MARCKS-like protein	0.6	0.7		1.6

MMP10	matrix metalloproteinase 10 preproprotein	4.9	2.9	3.9	
MNS1	meiosis-specific nuclear structural protein 1	0.5	0.6		1.8
MRPS6;SLC5A3	mitochondrial ribosomal protein S6;solute carrier family 5 (inositol transporters), member 3	0.6	0.6		1.6
MSN	moesin	1.7	1.5	1.6	
MT1E;MT1A;MT2A;MT1K	metallothionein 1K;metallothionein 1E;metallothionein 1A;metallothionein 2A	1.9	1.5	1.7	
MT2A;MT1K;MT1E;MT1A	metallothionein 2A;metallothionein 1K;metallothionein 1E;metallothionein 1A	1.9	1.5	1.7	
NAB1	NGFI-A binding protein 1	1.7	1.5	1.6	
NDRG1	N-myc downstream regulated gene 1	3.3	1.9	2.6	
NEDD9	neural precursor cell expressed, developmentally down-regulated 9	1.7	1.6	1.7	
NEXN	nexilin (F actin binding protein)	1.9	1.7	1.8	
NFKBIA	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	0.6	0.6		1.6
NPC1	Niemann-Pick disease, type C1	1.8	1.6	1.7	
NR3C1	nuclear receptor subfamily 3, group C, member 1 (glucocorticoid receptor)	1.7	1.5	1.6	
NR4A1	nuclear receptor subfamily 4, group A, member 1 isoform b;nuclear receptor subfamily 4, group A, member 1 isoform a	12.0	2.1	7.0	
NR4A2	nuclear receptor subfamily 4, group A, member 2	5.3	3.1	4.2	
NRIP1	receptor interacting protein 140	2.0	2.0	2.0	
NT5E	5' nucleotidase, ecto	3.5	1.9	2.7	
PALM2;AKAP2	A-kinase anchor protein 2 isoform 1;paralemmin 2;A-kinase anchor protein 2 isoform 2	1.9	1.8	1.8	
PBEF1	pre-B-cell colony enhancing factor 1 isoform b;pre-B-cell colony enhancing factor 1 isoform a	1.6	2.0	1.8	
PDE8A	phosphodiesterase 8A	1.6	1.6	1.6	
PDZK1	PDZ domain containing 1	0.5	0.5		2.0
PEG10	paternally expressed 10	0.5	0.5		1.9
PHLDB2	pleckstrin homology-like domain, family B, member 2	1.5	1.5	1.5	
PLAU	plasminogen activator, urokinase	2.8	2.3	2.6	
PLAUR	plasminogen activator, urokinase receptor	2.3	2.2	2.2	
PLEKHC1	pleckstrin homology domain containing, family C (with FERM domain) member 1	1.7	1.7	1.7	
PODXL	podocalyxin-like precursor	0.5	0.6		1.9
PTGS2	prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	7.1	5.4	6.3	
PTHLH	parathyroid hormone-like hormone isoform 2 preproprotein;parathyroid hormone-like hormone isoform 1 preproprotein	6.3	3.5	4.9	
PTPRE	protein tyrosine phosphatase, receptor type, E isoform 1 precursor;protein tyrosine phosphatase, receptor type, E isoform 2	3.1	2.6	2.9	

PTPRR	protein tyrosine phosphatase, receptor type, R isoform 2;protein tyrosine phosphatase, receptor type, R isoform 1 precursor	2.4	1.5	2.0	
QPRT	quinolinate phosphoribosyltransferase	0.5	0.5		1.9
RAI14	retinoic acid induced 14	1.9	1.6	1.7	
RAI3	retinoic acid induced 3	2.3	2.6	2.5	
RGC32	response gene to complement 32	2.5	2.0	2.2	
RHOF	ras homolog gene family, member F	2.1	2.0	2.1	
RHOU	ras homolog gene family, member U	0.4	0.5		2.2
S100A14	S100 calcium binding protein A14	2.1	1.6	1.9	
SAMD4	sterile alpha motif domain containing 4	2.4	1.5	1.9	
SARG	specifically androgen-regulated protein	3.9	1.7	2.8	
SCA1	ataxin 1	2.0	2.4	2.2	
SCEL	sciellin isoform b;sciellin isoform a	1.8	1.7	1.8	
SCHIP1	schwannomin interacting protein 1	2.1	1.9	2.0	
SDC4	syndecan 4 precursor	2.3	2.2	2.2	
SEC14L1	SEC14 (S. cerevisiae)-like 1	1.7	1.7	1.7	
SELK	selenoprotein K	2.1	1.6	1.9	
SEMA3B	semaphorin 3B	1.6	1.6	1.6	
SEPP1	selenoprotein P precursor	0.4	0.6		2.1
SEPT10	septin 10 isoform 1;septin 10 isoform 2	1.8	1.9	1.9	
SERPINE1	plasminogen activator inhibitor-1	5.6	2.1	3.9	
SFN	stratifin	2.0	1.7	1.9	
SH2D3A	SH2 domain containing 3A	2.2	1.5	1.9	
SH3MD2	SH3 multiple domains 2	1.6	1.5	1.6	
SLC20A1	solute carrier family 20 (phosphate transporter), member 1	2.0	1.7	1.8	
SLC20A2	solute carrier family 20, member 2	2.0	1.8	1.9	
SLC30A1	solute carrier family 30 (zinc transporter), member 1	1.8	1.9	1.9	
SLC37A1	solute carrier family 37 (glycerol-3-phosphate transporter), member 1	1.9	1.7	1.8	
SLC39A8	solute carrier family 39 (zinc transporter), member 8	2.2	1.8	2.0	
SLITRK5	SLIT and NTRK-like family, member 5	0.4	0.6		2.0
SLPI	secretory leukocyte protease inhibitor precursor	2.7	1.6	2.1	

SMURF2	E3 ubiquitin ligase SMURF2	1.6	1.5	1.6	
SOX17	SRY-box 17	3.6	2.4	3.0	
SOX4	SRY (sex determining region Y)-box 4	0.6	0.6		1.7
SPRY2	sprouty 2	2.6	2.0	2.3	
SRF	serum response factor (c-fos serum response element-binding transcription factor)	2.1	1.6	1.9	
SSFA2	sperm specific antigen 2	2.0	1.9	2.0	
STC1	stanniocalcin 1	2.3	1.7	2.0	
STK17A	serine/threonine kinase 17a (apoptosis-inducing)	1.7	1.6	1.7	
STK38L	serine/threonine kinase 38 like	2.1	1.8	1.9	
SYTL2	synaptotagmin-like 2	1.8	1.6	1.7	
TAGLN	transgelin	2.2	1.7	2.0	
TAZ	transcriptional co-activator with PDZ-binding motif (TAZ)	2.1	2.1	2.1	
TGFA	transforming growth factor, alpha	1.9	1.9	1.9	
TGFB3	transforming growth factor, beta 3	1.8	2.1	1.9	
TGFBI	transforming growth factor, beta-induced, 68kDa	2.1	2.1	2.1	
THBS1	thrombospondin 1 precursor	4.3	1.6	3.0	
THY28	thymocyte protein thy28 isoform 2;thymocyte protein thy28 isoform 1	0.6	0.6		1.6
TM4SF1	transmembrane 4 superfamily member 1	6.2	1.7	4.0	
TMEM16A	transmembrane protein 16A	2.0	1.9	1.9	
TMEM22	transmembrane protein 22	1.9	1.6	1.8	
TMEPAI	transmembrane prostate androgen-induced protein	2.9	1.9	2.4	
TMPO	thymopoietin	0.6	0.6		1.6
TMPRSS2	transmembrane protease, serine 2	0.3	0.6		2.1
TMSNB	thymosin, beta, identified in neuroblastoma cells	0.6	0.6		1.7
TNFAIP1	tumor necrosis factor, alpha-induced protein 1	2.6	1.6	2.1	
TNFAIP8	tumor necrosis factor, alpha-induced protein 8	0.4	0.6		2.0
TNFRSF12A	type I transmembrane protein Fn14	1.9	2.0	2.0	
TNFRSF6	tumor necrosis factor receptor superfamily, member 6	2.7	1.8	2.3	
TNFSF9	tumor necrosis factor (ligand) superfamily, member 9	2.5	1.8	2.2	
TNNC1	troponin C, slow	3.2	2.4	2.8	

TPM4	tropomyosin 4	2.0	1.7	1.8	
TRAF4	TNF receptor-associated factor 4 isoform 2;TNF receptor-associated factor 4 isoform 1	1.6	1.5	1.5	
TRHDE	thyrotropin-releasing hormone degrading ectoenzyme	3.2	2.4	2.8	
TRIB1	G-protein-coupled receptor induced protein	3.0	2.4	2.7	
TRIB3	tribbles 3	2.3	1.8	2.0	
TRIM6	tripartite motif protein 6	0.5	0.5		2.1
TXNIP	thioredoxin interacting protein	0.2	0.6		2.4
TXNRD1	thioredoxin reductase 1	2.2	2.0	2.1	
UBTF	upstream binding transcription factor, RNA polymerase I	0.2	0.5		2.8
UCP2	uncoupling protein 2	0.6	0.6		1.6
UPF3B	UPF3 regulator of nonsense transcripts homolog B isoform 2;UPF3 regulator of nonsense transcripts homolog B isoform 1	0.6	0.6		1.6
VIL2	villin 2	1.9	1.8	1.8	
WNT11	wingless-type MMTV integration site family, member 11 precursor	0.5	0.6		1.8
ZNF165	zinc finger protein 165	1.6	1.6	1.6	
ZNF488	zinc finger protein 488	0.3	0.6		2.2

Table 12. Treatment of PROKR1 Ishikawa cells with 40nM PROK1 revealed 49 genes to be regulated with fold changes (expressed relative to vehicle treated PROKR1 cells) of greater than 1.5 on both gene arrays after application of selection criteria of adjusted p values of less than or equal to 0.05 on either array. Analysis revealed 46 genes to be up-regulated by greater than 1.5 fold, while 3 genes were revealed to be down-regulated (indicated by -) by greater than 1.5 fold.

Gene Symbol	Gene Product	mean fold change
ACSL4	acyl-CoA synthetase long-chain family member 4 isoform 2	1.5
AKAP12	A-kinase anchor protein 12 isoform 2	2.8
AREG	amphiregulin preproprotein	5.3
BNC1	basonuclin 1	2.4
CD44	CD44 antigen isoform 4 precursor	1.9
CORO1C	coronin	2.3
DAF	decay accelerating factor for complement	9.8
DCAMKL1	doublecortin and CaM kinase-like 1	2.6
DKK1	dickkopf homolog 1	8.5
DNAJB9	DnaJ (Hsp40) homolog, subfamily B, member 9	1.9
DSCR1	calcipressin 1 isoform c	5.2
DTR	diphtheria toxin receptor/ heparin binding EGF-like growth factor	3.6
DUSP1	dual specificity phosphatase 1	4.4
DUSP14	dual specificity phosphatase 14	1.9
DUSP4	dual specificity phosphatase 4 isoform 2	9.4
DUSP5	dual specificity phosphatase 5	7.2
EGR1	early growth response 1	2.1
EIF2AK3	eukaryotic translation initiation factor 2-alpha kinase 3	1.7
ENAH	enabled homolog	1.7
FGD6	RhoGEF and PH domain containing 6	1.8
GEM	GTP-binding mitogen-induced T-cell protein	5.3
GREM1	cysteine knot superfamily 1	12.4
HERC4	hect domain and RLD 4	1.9
HMGB2	high-mobility group box 2	-1.6
IER3	immediate early response 3	9.2
IL11	interleukin 11 precursor	19.8
IL6	interleukin 6	17.2
IL8	interleukin 8 precursor	9.7
KRTHA4	type I hair keratin 4	5.8
LAMA3	laminin alpha 3 subunit isoform 2	2.0
LIF	leukemia inhibitory factor	2.6
MAIL	molecule possessing ankyrin repeats induced by lipopolysaccharide	3.6
NR3C1	nuclear receptor subfamily 3	1.6
NR4A1	nuclear receptor subfamily 4, group A, member 1 isoform	7.0
NR4A2	nuclear receptor subfamily 4, group A, member 2 isoform	4.3
PBEF1	pre-B-cell colony enhancing factor 1 isoform	1.8
PTGS2	prostaglandin-endoperoxide synthase 2	6.3
PTPRR	protein tyrosine phosphatase, receptor type, R	2.0
SCHIP1	schwannomin interacting protein 1	2.0
SERPINE1	plasminogen activator inhibitor-1	3.9
SLC20A2	solute carrier family 20, member 2	1.9
STK38L	serine/threonine kinase 38 like	1.9
TMEM22	transmembrane protein 22	1.8
TMPRSS2	transmembrane protease, serine 2	-2.1
TNFAIP1	tumor necrosis factor, alpha-induced protein 1	2.3
TRIB1	G-protein-coupled receptor induced protein	2.7
TRIB3	tribbles 3	2.0
TXNIP	thioredoxin interacting protein	-3.0
ZNF165	zinc finger protein 165	1.6

Table 13. Treatment of PROKR1 Ishikawa cells with 40nM PROK1 for 8 hours revealed 277 genes to be regulated. Application of gene ontology annotation for biological processes to PROK1 regulated genes revealed 3 significant themes of cellular morphogenesis, cell motility/chemotaxis and blood vessel development/morphogenesis. GO numbers are numbers assigned to a biological function within the gene ontology database. Numbers within brackets denote the number of genes from the list of genes regulated by PROK1 within that category.

Cell morphogenesis/developmental processes	
GO:0032989	cell structure morphogenesis (16)
GO:0030154	cell differentiation (54)
GO:0009887	organ morphogenesis (20)
GO:0002009	morphogenesis of an epithelium (7)
GO:0051017	actin filament bundle formation (4)
GO:0007015	actin filament organisation (5)
GO:0003779	actin binding (12)
GO:0048646	anatomical structure formation (13)
GO:0009653	anatomical structure morphogenesis (41)
GO:0048856	anatomical structure development (70)
Cell motility/chemotaxis	
GO:0006928	cell motility (18)
GO:0007626	locomotory behaviour (11)
GO:0051270	regulation of cell motility (6)
GO:0042330	chemotaxis (10)
Blood vessel development /morphogenesis	
GO:0035295	tube development (9)
GO:0001525	angiogenesis (13)
GO:0048514	blood vessel morphogenesis (14)
GO:0001568	blood vessel development (15)
GO:0009611	response to wounding (22)
GO:0042060	wound healing (8)
GO:0001944	vasculature development (17)
GO:0001568	blood vessel development (17)
GO:0001525	angiogenesis (15)
GO:0045765	regulation of angiogenesis (3)

5.3.3. Validation of gene expression implicated by gene array

5.3.3.1. Validation of gene expression at 8 hours

As discussed earlier in section 5.1, there are always concerns regarding the validity of results obtained purely from global gene array analysis of gene expression. Therefore, in order to verify the data suggested by gene array analysis, five genes were selected for further investigation to confirm their regulation by PROK1. COX-2, LIF, IL-6, IL-8 and IL-11 were selected for further examination by Taqman quantitative PCR analysis. PROKR1 and WT Ishikawa cells were treated with 40nM PROK1 or vehicle for 8 hours as this would enable direct comparisons to be drawn with the data obtained from the gene array analysis. RNA was extracted from the cells following treatment with vehicle or 40nM PROK1 and subjected to Taqman quantitative PCR analysis for COX-2, LIF, IL-6, IL-8 and IL-11. Data are expressed as fold above vehicle treated controls. Taqman quantitative PCR analysis of these genes confirmed them to be elevated upon treatment with 40nM PROK1 for 8 hours. Fold induction of gene expression by 40nM PROK1 at 8 hours in PROKR1 Ishikawa cells (Figure 5.1) was as follows: COX-2 (4.82 ± 1.2 fold), LIF (12.4 ± 3.4 fold), IL-6 (15.55 ± 1.8 fold), IL-8 (152 ± 81.14 fold) and IL-11 (249.6 ± 88.3 fold). However, no significant elevation of gene expression was observed in WT Ishikawa cells following treatment with 40nM PROK1 for any of the genes examined (Figure 5.1).

5.3.3.2. Temporal regulation of PROK1 induced gene expression

PROK1 regulated expression of COX-2, LIF, IL-6, IL-8 and IL-11 has been demonstrated in PROKR1 and WT Ishikawa cells at 8 hours. This validates gene expression suggested by global gene array analysis. Subsequently the temporal regulation of PROK1 induced gene expression was examined in order to determine the time of maximal expression for each gene. PROKR1 and WT Ishikawa cells were treated with vehicle or 40nM PROK1 for 0, 2, 4, 6, 8, 12 and 24 hours. RNA was extracted and subjected to Taqman quantitative PCR analysis. From these data it is observed that LIF (red bars) and COX-2 (blue bars) are maximally expressed at 6 hours, IL-6 (yellow bars) and IL-11 (turquoise bars) are maximally expressed at 8

hours and IL-8 (green bars) is maximally expressed at 8-12 hours in PROKR1 Ishikawa cells. No elevation of gene expression in response to PROK1 was observed in WT Ishikawa cells (grey bars) at any time (Figure 5.2).

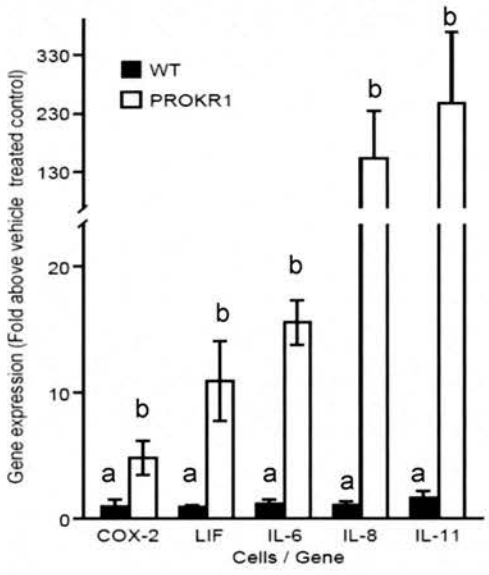


Figure 5.1. PROK1 mediated gene expression at 8 hours. WT and PROKR1 Ishikawa cells were treated with 40nM PROK1 or vehicle for 8 hours. Gene expression was assessed by Taqman PCR analysis for COX-2, LIF, IL-6, IL-8 and IL-11. Data are expressed as fold above vehicle treated control. Data are presented as mean ± SEM. (b is significantly different from a $p<0.05$).

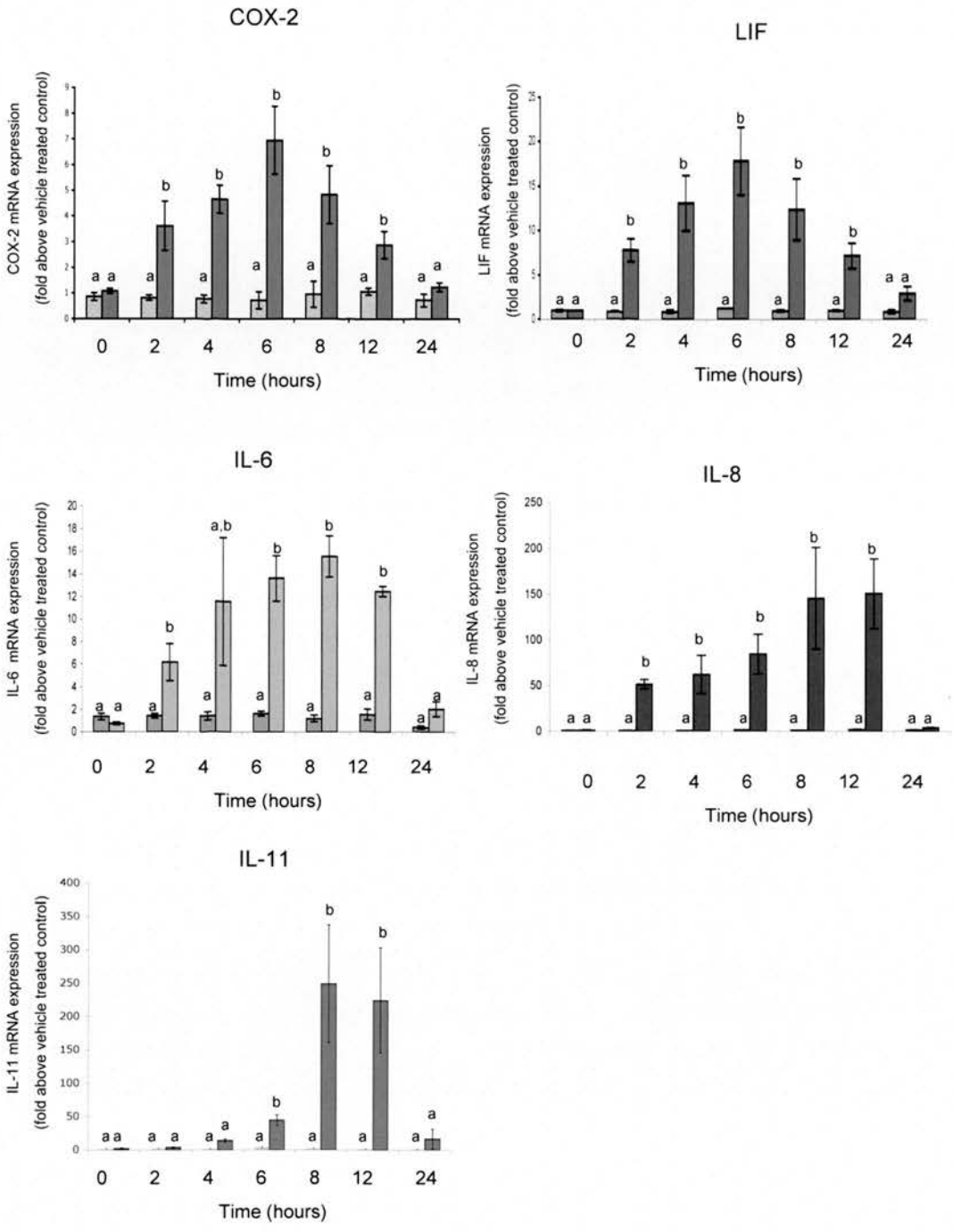


Figure 5.2. Temporal regulation of PROK1 induced gene expression. PROKR1 and WT Ishikawa cells were treated with 40nM PROK1 or vehicle for 0, 2, 4, 6, 8, 12 and 24 hours. Gene expression was assessed by Taqman quantitative PCR analysis for COX-2. LIF, IL-6, IL-8 and IL-11. Data are expressed as fold above vehicle treated control. No elevation of gene expression in response to 40nM PROK1 was observed in WT Ishikawa cells (grey bars) at any time point. However, there was significant elevation of IL-8 (green bars), IL-6 (yellow bars), IL-11 (turquoise bars), LIF (red bars) and COX-2 (blue bars). Data are presented as mean ± SEM. (b is significantly different from b $p<0.05$)

5.4. Discussion

In chapter 3 it was demonstrated that PROK1 is elevated during the mid-secretory phase of the menstrual cycle. Due to elevation during this phase of the menstrual cycle it was anticipated that PROK1 may be involved in endometrial receptivity and implantation. A number of proteins which are elevated during this phase of the menstrual cycle are proposed to play a role in endometrial receptivity, and one strategy prior to performing a microarray experiment could have been choosing a number of targets to investigate by real time PCR. However, as there was no literature concerning gene regulation by PROK1 in the endometrium or any other tissues, this approach seemed somewhat haphazard and may not have yielded any data. Additionally, although PROK1 is elevated during the mid-secretory phase of the menstrual cycle, this elevation was not significantly different to the elevation observed during the early- or late-secretory phase, therefore focussing effort on determining potential regulation of genes solely during the mid-secretory phase of the menstrual cycle may have excluded potentially interesting genes which are regulated during the early- and late secretory phase of the cycle.

An added complication is that, in examining gene arrays performed on endometrium collected across the menstrual cycle, there is surprisingly little consensus in genes regulated. As indicated in Table 1, out of 6 gene array analyses performed on endometrial tissue comparing receptive with non-receptive endometrium, only 26 genes were reported as regulated in a concordance of 3 array analyses. This is a surprisingly low number considering the high number of genes reported to be regulated in the individual gene arrays. Again, these data represent mainly genes regulated in the receptive endometrium during the mid-secretory phase of the menstrual cycle; little data exists comparing multiple phases of the menstrual cycle excepting a recent report by Talbi *et al* (2006). It appeared that microarray analysis would give the most meaningful, global indication of genes regulated by PROK1 in the endometrium as this wouldn't simply focus on a single phase of the menstrual cycle.

In order to investigate gene activation initiated upon PROK1-PROKR1 interaction global gene array analysis was performed. Two different platforms, the Affymetrix GeneChip® Human Genome U133 plus 2.0 and the AB1700 v.2

Applied Biosystems Human Genome Survey microarrays, were used in this study. Concordance between the two platforms in significantly changed genes were extracted from the data. With selection criteria of genes changed by 1.5 fold or greater on both platforms applied to the data, 277 genes were found to be differentially regulated. Application of more stringent criteria with a fold change of 1.5 or more on both platforms and an adjusted p value of <0.05 on either platform revealed 49 genes to be differentially regulated by PROK1.

Initially, in order to validate the gene array data, five genes were chosen and the level of expression induced by PROK1 in PROKR1 and WT Ishikawa cells examined at 8 hours by real-time PCR. These data confirmed the increase in expression observed in the arrays for COX-2 (4.8 fold), LIF (12.4 fold), IL-6 (15.55 fold), IL-8 (152 fold) and IL-11 (249.6 fold). No increase in gene expression was detected in WT Ishikawa cells. The large difference in fold change seen with IL-8 and IL-11 between array versus PCR analysis is likely due to the smaller dynamic range of the gene array at large fold changes compared to high dynamic range of PCR based analysis. Time course analysis of the effect of PROK1 on expression of the five chosen genes revealed maximal expression of IL-8 at 8-12 hours, IL-6 at 8 hours, IL-11 at 8 hours, LIF at 6 hours and COX-2 at 6 hours. These data may indicate that, for a number of genes, 8 hours may not be the maximal time point.

The genes chosen for further investigation were LIF and COX-2. These genes were chosen as being potentially interesting when considering a potential role for PROK1 signalling the receptivity and implantation. Although previous reports disagree on the stage of the menstrual cycle at which maximal COX-2 expression is observed gene knockout studies have implicated a role for COX-2 in receptivity and decidualization, this led to the interest in the regulation of COX-2 expression in the endometrium and decidua by PROK1. LIF expression is elevated during the mid-secretory phase of the menstrual cycle, and although its role in human reproduction has not yet been defined, gene knockout studies have demonstrated an absolutely essential role for this cytokine in implantation in mice. COX-2 and LIF were therefore chosen as interesting candidate genes to follow up with reference to a potential role for PROK1 in receptivity and implantation.

Analysis of gene ontology (GO) annotations for genes regulated by PROK1 revealed three major themes representing biological processes controlled by PROK1 regulated genes. These 3 areas were cell morphogenesis, cell motility/chemotaxis and blood vessel development/morphogenesis. These processes are highly significant with reference to endometrial function specifically at the time when PROK1 is elevated during the mid-secretory phase of the menstrual cycle and during early pregnancy. These processes are instrumental in the endometrium in preparation for and during early pregnancy when angiogenesis, stromal cell decidualization and cell migration occur.

A large number of genes elevated by PROK1 upon PROKR1 activation have been implicated in implantation. Some of the best examples, with data provided from knockout studies, *in vitro* studies on human tissue and expression analysis in endometrial tissue across the menstrual cycle include IL-11, LIF, COX-2, HB-EGF (DTR) and DAF (decay accelerating factor for complement). The genes regulated by PROK1 also demonstrate some overlap with genes regulated during the window of implantation demonstrated by gene arrays conducted on endometrium taken during the mid-secretory phase of the menstrual cycle. These genes include DAF, Laminin, serine protease inhibitors, Dkk-1, protein tyrosine phosphatase receptors, LIF, THBS1, metallothioneins, TGF-β superfamily, serine/threonine kinases, LDL receptor protein and GADD45A (growth arrest and DNA damage inducible protein, DNA excision repair and cell-cycle regulation) (Kao *et al*, 2002, Carson *et al*, 2002, Riesewijk *et al*, 2003, Borthwick *et al*, 2003, Mirkin *et al*, 2005, Talbi *et al*, 2006).

Division of PROK1 regulated genes into GO classifications of cell morphogenesis; cell motility/chemotaxis and blood vessel development/morphogenesis allows discussion of the genes within functional groups. A number of genes which were elevated by PROK1 and which may play functional roles in the endometrium in preparation for or during implantation, will be discussed further. PROK1 induced genes may be represented in more than one functional group. Genes will therefore be discussed within the relevant functional groups with a review of these functions.

5.4.1. GO - cell morphogenesis/developmental processes

A large number of genes elevated by PROK1 within the GO classification for cell morphogenesis/developmental processes are relevant to implantation.

Basonuclin-1 is a cell specific transcription factor; its functions include re-epithelialisation (Matsuazki *et al*, 2004) and cell adhesion (Wang J *et al*, 2006). Regulation by PROK1 may indicate a role for Basonuclin-1 in blastocyst adhesion and repair of the luminal epithelium after rupture and invasion by the trophoblast. As PROK1 is elevated in first trimester decidua, this may also imply a prolonged role for Basonuclin-1 in repair of areas breached by the invading trophoblast in the aggressively invasive stage of the first trimester.

Migration of extravillous trophoblast cells mediated by PGE_2 is dependent on proteases called Calpains (Nicola *et al*, 2005). These Calpains are calcium dependent proteases involved in cell detachment from the substratum during migratory responses (Dourdin *et al*, 2001, Huttenlocher *et al*, 1997, Glading *et al*, 2000). VEGF also mediates angiogenic effects via induction of Calpain 2 (Su Y *et al*, 2006). Therefore in initiation of an endometrial - trophoblast dialogue, PROK1 induced Calpain proteases may aid developmental processes during the early stages of placentation.

Tissues, such as the invading trophoblast, undergoing rapid growth and regeneration contain hyaluronic acid (Goshen *et al*, 1996), which mediates its effect through its major cell surface receptor CD44 (Sherman *et al*, 1994). CD44 is suggested to be elevated in the epithelial cells during the window of implantation (Horne *et al*, 2002, Afify *et al*, 2006), and may contribute to the edema observed in the endometrium at this phase of the menstrual cycle (Okada *et al*, 2001). CD44 is also suggested to be involved in trophoblast attachment and implantation (Aplin, 1997, Goshen *et al*, 1996, Albers *et al*, 1995). PROK1 via elevation of CD44 may therefore play dual roles in endometrial edema and blastocyst attachment.

The α-crystallin B-chain first appears in the early secretory phase of the menstrual cycle and continues to rise until the late secretory phase (Gruidl *et al*, 1997). It localises to the luminal and glandular epithelium and is controlled by progesterone (Gruidl *et al*, 1997). α-crystallin B-chain is also elevated in the baboon endometrium upon treatment with hCG, one of the first embryonic products, potentially indicating, along with the temporal expression and localization in the human endometrium a role in implantation (Sherwin *et al*, 2007). α-crystallin B-chain is suggested to confer resistance against cytotoxicity induced by TNF-α (suggested to be involved in trophoblast invasion) and oxidative stress at the time of implantation (Mehlen *et al*, 1995).

A family of proteins induced by PROK1, and related to α-crystallin B-chain, are the heat shock proteins. These are stress induced proteins. They are expressed in the endometrium and HSP27 (heat shock protein 27 or B8) increases rapidly after ovulation (Tabibzadeh *et al*, 1996) and are present in the endometrium until week 10 of pregnancy (Padwick *et al*, 1994). Like α-crystallin B-chain, HSP's are thought to confer resistance against cytotoxicity and oxidative stress (Melen *et al*, 1995). PROK1 may therefore act as a protective factor, possibly during the hypoxic phase of placentation via induction of HSP family members.

DCAMKL-1 (doublecortin and CaM kinase like-1) regulates cell migration and development (Friocourt *et al*, 2003). These processes are essential to the trophoblast during implantation and invasion. However, a role in migration has thus far been demonstrated solely in neurones (Friocourt *et al*, 2003). Expression of DCAMKL-1 is also thought to link calcium signalling and microtubule dynamics during migration (Lin PT *et al*, 2000). PROK1 mediated signalling appears to be calcium dependent (Soga *et al*, 2002, Masuda *et al*, 2002). Therefore PROK1 mediated signalling via DCAMKL-1 and calcium activation may indeed be of relevance to cell migration in the endometrium. There are currently no reports of DCAMKL-1 expression in the endometrium. However, if a communication between the developing foetus and the endometrium is initiated, PROK1 mediated DCAMKL-1 may play a role in neuronal migration in the fetal brain (Mizuguchi *et al*, 1999, Qin *et al*, 2000).

Dkk-1 (Dickkopf) is a Wnt antagonist and is structurally related to PROK1 (LeCouter *et al*, 2001). Dkk-1 is elevated during the window of implantation in a number of gene array analyses examining endometrium taken during this phase of the menstrual cycle (Kao *et al*, 2002, Carson *et al*, 2002, Riesewijk *et al*, 2003, Borthwick *et al*, 2003, Mirkin *et al*, 2005), is estrogen and progesterone responsive (Tulac *et al*, 2006) and peaks in the mid-secretory phase of the menstrual cycle. As a Wnt antagonist Dkk-1 may limit Wnt actions during the establishment of endometrial receptivity to implantation and the early stages of invasion (Tulac *et al*, 2006). It is proposed that endometrial expression of Dkk-1 may signal the opening of the implantation window (Zhang *et al*, 2004), potentially implicating PROK1 in implantation related functions.

HB-EGF (heparin binding epidermal growth factor) is also known as DTR (diphtheria toxin receptor) as the transmembrane form of HB-EGF actually acts as the receptor for diphtheria toxin (Raab *et al*, 1994, Naglich *et al*, 1992, Iwamoto *et al*, 1994). Expression of HB-EGF in the endometrium has been heavily implicated in implantation. In the mouse HB-EGF is expressed on the luminal epithelium 6 hours prior to the attachment reaction between the epithelium and the blastocyst (Das *et al*, 1994), while EGFR is expressed on the trophectoderm (Chobotova *et al*, 2002b, Dardik *et al*, 1992). In the LIF null mouse, which does not display strong blastocyst - endometrial attachment or implantation, HB-EGF is not expressed at the luminal epithelium at the site of blastocyst apposition on day 4 or 5 (Song *et al*, 2000). Cells expressing transmembrane HB-EGF can adhere to active but not dormant blastocysts *in vitro* (Raab *et al*, 1996). It is therefore suggested that transmembrane HB-EGF induced at the site of blastocyst apposition may mediate attachment. A role for HB-EGF has also been suggested in the human endometrium. HB-EGF is expressed in the human endometrium during the window of implantation (Leach *et al*, 1999, Yoo *et al*, 1997). Its expression coincides with the appearance of pinopodes, with expression of HB-EGF on the surface of the pinopodes (Stavreus-Evers *et al*, 2002). Pinopodes are protrusions, which are thought to be essential to implantation (Pantos *et al*, 2004). HB-EGF is also suggested to improve blastocyst development *in vitro* (Martin *et al*, 1998) and adheres to human blastocysts expressing the EGFR ErbB4 (Chobotova *et al*,

2002b) and heparin sulphate proteoglycan (Paria *et al*, 1999). HB-EGF is also suggested to have a role in decidualisation as inhibition of HB-EGF inhibits decidualisation and survival of stromal cells (Chobotova *et al*, 2002a). TGF-β, which is also regulated by PROK1, has been demonstrated to induce expression of transmembrane HB-EGF (Chobotova *et al*, 2002a). PROK1 may therefore regulate HB-EGF expression directly and perpetuate expression though induction of TGF-β.

Follistatin is an Activin A binding protein. Activin A promotes decidualisation (Jones *et al*, 2002, Tierney and Giudice, 2004), which is inhibited by Follistatin. Activin A produced by decidualized stromal cells may augment invasion and placental function; follistatin may serve to limit the Activin A mediated trophoblast invasion and prevent excessive invasion (Caniggia *et al*, 1997). The temporal regulation of Follistatin supports this, as along with Activin A, it is produced in the decidualized stromal cells of secretory endometrium and early pregnancy (Jones *et al*, 2002), potentially suggesting paracrine regulation.

TGF-β has similar effects to another member of the TGF superfamily, Follistatin, described above. TGF-β inhibits trophoblast growth (Li RH *et al*, 1997) invasion and proliferation (Graham and Lala, 1991, Graham, 1997, Morrish *et al*, 1991, Graham, 1992 *et al*, Li & Zhang, 1997, Smith, 2001). TGF-β3 is over-expressed in pre-eclamptic pregnancies (Caniggia *et al*, 1999) where insufficient trophoblast invasion and deficient placentation are markers of this pathology. Expression of TGF-β3 varies across the menstrual cycle with elevation and intense glandular epithelial expression in the secretory phase of the menstrual cycle (Reis *et al*, 2002, Tang *et al*, 1994). Activation may release from the latent form by uPA (Casslen *et al*, 1998), TGF-β as indicated above may act in a negative feedback mechanism to inhibit excessive invasion. Elevation of both Follistating and TGF-β3 by PROK1 may implicate a role for PROK1-PROKR1 signalling in limiting trophoblast invasion.

IL-11 is a member of the IL-6 family and its signalling has been heavily implicated in early pregnancy. Using knockout mice for the IL-11 receptor α (IL-11Rα) it was demonstrated that blastocysts could implant. However an

insufficient decidualisation reaction was initiated in response to the blastocyst. This was shown to be a maternal defect as 2.5 day embryos transferred from the IL-11Rα null mice to WT mice implanted and survived, while transfer of 2.5 day embryos from WT mothers to IL-11Rα null mice again displayed an insufficient decidualisation reaction (Robb *et al*, 1998). These data indicate that endometrial IL-11 signalling is essential for proper decidualisation (Robb *et al*, 1998, Bilinski *et al*, 1998). In order for proper signalling to occur, IL-11 must induce formation of a heterodimeric complex of its own receptor with the common IL-6 family receptor gp130. Similarly gp130 null mice display intrauterine defects (Ernst *et al*, 2001). IL-11 and its receptor have been identified in decidual cells in the secretory phase of the menstrual cycle and early pregnancy (Chen HF *et al*, 2002, Cork *et al*, 2002, Dimitriadis *et al*, 2002, 2003, Karpovich *et al*, 2003). The receptors IL-11Rα and gp130 are expressed on the glandular and luminal epithelium (Cullinan *et al*, 1996, Cork *et al*, 2002, Van Rango *et al*, 2004) with expression also noted in trophoblast cells (Dimitriadis *et al*, 2003). This could imply a signalling dialogue for IL-11 between the endometrium and trophoblast. The observation that IL-11 expression is reduced in anembryonic pregnancies which result in early abortion (Chen HF *et al*, 2002), adds weight to the proposed involvement of IL-11 signalling in implantation and decidualisation.

Expression of Galectins has been detected in the endometrium (Koopman, 2003 *et al*, Maquoi *et al*, 1997). Galectins are a group of soluble lectins, which are thought to mediate trophoblast attachment (Kimber & Spanswick, 2002). Galectin-1 is proposed to mediate interactions between integrins and ECM components, which are of vital importance in endometrium-blastocyst attachment (Gu *et al*, 1994, Maquoi *et al*, 1997). PROK1 may therefore play a role in blastocyst adhesion.

LIF (leukaemia inhibitory factor) has been demonstrated by use of LIF null mice to be absolutely essential in implantation as they do not display any signs of implantation and cannot support pregnancy (Stewart *et al*, 1992). LIF is also a member of the IL-6 family of cytokines and could possibly substitute for IL-6 in the IL-6 null animals during implantation (Robertson *et al*, 2000).

Expression of endometrial LIF is stimulated by HB-EGF and TGF-α (Arici *et al*, 1995). As PROK1 up-regulates expression of these factors as well as LIF this may provide a mechanism for prolonged LIF expression in the endometrium. The role of LIF will be discussed more fully in chapter 7.

Expression of IL-8 has been demonstrated in the secretory endometrium (Milne *et al*, 1999, Jones *et al*, 1997, Critchley *et al*, 1994, Arici *et al*, 1998). IL-8 enhances adhesion of cells to fibronectin, a component of the extracellular matrix (Garcia-Velasco *et al*, 1999, Mulayim *et al*, 2004). These data suggest a role for IL-8, and therefore PROK1, in the adhesion of the trophoblast in early pregnancy.

IL-6, along with its family members, LIF and IL-11, has also been implicated in implantation. IL-6 null mice can implant and produce live offspring however, they display a 48% reduction in implantation compared with their WT counterparts (Robertson *et al*, 2000), and this may indicate a degree of redundancy for IL-6 as it may be compensated for by other members of the IL-6 family. In the human endometrium IL-6 is present in the mid-secretory endometrium localised to the luminal and glandular epithelium (Tabibzadeh, 1995 *et al*, Vandermolen & Gu, 1996). IL-6 is suggested to contribute to trophoblast growth and placental development in humans (Nishino *et al*, 1990). IL-6 receptors are present in the endometrium and trophoblast during implantation and placentation (Deb *et al*, 1999, Nishino *et al*, 1990, Masuhiro *et al*, 1991, Li Y *et al*, 1992) and via interaction with the trophoblast IL-6 elevates secretion of hCG (Matsuzaki *et al*, 1995). IL-6 may therefore contribute to the growth and differentiation of the trophoblast in early pregnancy.

PTGS2 or COX-2 (cyclooxygenase-2) has been demonstrated by the knockout mouse model for COX-2 to have an essential role in implantation events and decidualisation (Cheng & Stewart, 2003, Lim *et al*, 1997). COX-2 null mice display a delay in implantation, a defective decidual response and a delay in parturition. It has been demonstrated in humans that implantation past the normal window of implantation may leads to defective formation of the feto-

placental unit and the risk of pregnancy loss increases as a function of delaying the initiation of pregnancy (Red Horse *et al*, 2004, Wilcox *et al*, 1999). The role of COX-2 will be discussed in more detail in chapter 6.

TGF-α is present in the mouse endometrium throughout pregnancy and in human endometrium with a peak in the secretory phase of the menstrual cycle and is localised to the luminal epithelium (Ejskjaer *et al*, 2005). In human cells it elevates secretion of hCG from the trophoblast and enhances trophoblast growth (Edwards, 1995). It also elevates expression of HB-EGF (Chobotova *et al*, 2002a) and therefore contributes to decidualisation and stromal cell survival. PROK1 mediated TGF-α expression may therefore promote decidualization during early pregnancy.

5.4.2. GO - cell motility/ chemotaxis

A number of genes elevated by PROK1 are proposed to play important roles in cell motility and chemotaxis. These are highly important functions during the invasive phase of implantation and placentation.

CD44 can recruit proteolytically active MMP-7 and HB-EGF to form a cell surface complex, which may play a role in regulation of tissue remodelling (Yu WH *et al*, 2002), which may be important in trophoblast migration and invasion.

CTGF may have multiple roles in the endometrium. Connective tissue growth factor (CTGF) has been localised to the glandular and luminal epithelium and stroma in the secretory phase of the menstrual and decidua of early pregnancy in human and mouse (Uzumcu *et al*, 2000, Surveyor *et al*, 1998). It appears likely that CTGF is produced as a prelude or in consequence of decidualisation and is therefore thought to play a role in early pregnancy (Brigstock, 1999). Roles for CTGF have been suggested in uterine cell growth, migration, adhesion, and extracellular matrix production (Surveyor *et al*, 1998).

Cyr61 is a member of the same family as CTGF, the CCN family (an acronym for Connective tissue growth factor, Cysteine rich protein (Cyr61), and Nephroblastoma overexpressed gene). This family mediates diverse roles in development, cell proliferation and tumourigenesis with its expression rapidly induced by growth factors cytokines and estrogen (Brigstock, 2003, Kireeva *et al*, 1996). Cyr61 is present in baboon endometrium during the menstrual cycle and localises to the glandular and luminal epithelial cells of eutopic and ectopic endometrium during the secretory phase of the cycle (Gashaw *et al*, 2006). In the mouse Cyr61 localises to the luminal epithelium on day 4 at the time of implantation (Chen Y *et al*, 2006).

Expression of IL-8 has been localised to the luminal and glandular epithelial cells and activated macrophages of the secretory endometrium (Milne *et al*, 1999, Jones *et al*, 1997, Critchley *et al*, 1994, Arici *et al*, 1998). It is suggested that IL-8 as a chemokine, is involved in recruitment of neutrophils and leukocytes into the endometrium, large numbers of which are observed in the secretory phase of the cycle (Arici *et al*, 1998, Jones *et al*, 1997). IL-8 elevates activation of collagenases and matrix metalloproteinases (MMP's, Garcia-Velasco *et al*, 1999, Mulayim *et al*, 2004). These data may suggest a role for IL-8, and therefore PROK1, in the invasion of the trophoblast in early pregnancy.

Urokinase plasminogen activator (uPA/ PLAU) is a protease in its own right and also activates other proteases from their pro-forms e.g. the MMP's. PROK1 may induce expression of uPA in the endometrium and the cytotrophoblast also produces uPA (Queenan *et al*, 1987), therefore uPA is freely available at the invasion site for invasion of the trophoblast (Nordengren *et al*, 2004). As well as aiding invasion uPA may also act to inhibit excessive invasion. Release of TGF-β from its latent form by uPA (Odekon *et al*, 1994, Yee *et al*, 1993) enables active TGF-β to inhibit invasion, therefore uPA can possibly promote invasion and via TGF-β initiate a negative feedback mechanism to inhibit invasion. PROK1 also elevates the receptor for uPA (PLAUR) therefore enabling its action. However, PROK1 also elevated the inhibitor for uPA, SERPINE-1. Serine protease inhibitors have also been

demonstrated by gene array analysis to be elevated the in window of implantation (Kao, 2002 *et al*, Carson, 2002 *et al*, Riesewijk *et al*, 2003, Borthwick *et al*, 2003, Mirkin *et al*, 2005). The activity of pro- or anti-invasive mechanisms may therefore depend on the local levels of each or other regulators present in the endometrial milieu.

5.4.3. GO - blood vessel development/morphogenesis

Another group of PROK1 induced genes that are highlighted in the GO groupings are those affecting blood vessel development and morphogenesis. These gene products may contribute to endometrial vessel maturation during the secretory phase of the cycle or regulation of blood vessels during early pregnancy.

CD44, as indicated above, is implicated in cell morphogenesis. However, it also has potential roles in blood vessel development. During the secretory phase of the menstrual cycle CD44 localises to the perivascular cells and its expression is most intense adjacent to the spiral arterioles (Afify *et al*, 2006). This may suggest a role for CD44, and therefore PROK1, in endothelial cell support.

CTGF is mainly represented in GO annotations for blood vessel development and morphogenesis. In the pregnant human endometrium, CTGF localises to the vascular endothelial cells (Uzumcu *et al*, 2000). It appears likely that CTGF is produced as a prelude or in consequence of decidualisation and is therefore thought to play a role in early pregnancy (Brigstock, 1999), however this requires further investigation. The related proangiogenic factor Cyr61 is present in baboon endometrium during the menstrual cycle and localises to the blood vessels of eutopic and ectopic endometrium during the secretory phase of the cycle (Gashaw *et al*, 2006). Cyr61 has been demonstrated to potentiate the effects of the angiogenic factors FGF-2 and PDGF (Kireeva *et al*, 1996). PROK1 may therefore mediate physiological angiogenesis during early pregnancy.

DAF has been demonstrated to be elevated during the window of implantation in a number of gene array analyses examining endometrium taken during this phase of the menstrual cycle (Kao *et al*, 2002, Carson *et al*, 2002, Riesewijk *et al*, 2003, Borthwick *et al*, 2003, Mirkin *et al*, 2005). DAF localises to the luminal and glandular epithelium in cycling endometrium (Francis *et al*, 2006) and is dramatically elevated during the mid-secretory phase of the cycle (Young *et al*, 2002, Mirkin *et al*, 2005 *et al*, Talbi, 2006), during which time its expression may be stimulated by EGF family members (Young *et al*, 2002). However, expression of DAF is reduced in women exhibiting luteal phase defect, which is restored after treatment with progesterone (Kaul *et al*, 1995). Antiphospholipid syndrome (APS) is associated with placental insufficiency and a high miscarriage rate; DAF is significantly lower in these patients (Francis *et al*, 2006). This is thought to be due to the degree of cellular protection against complement mediated lysis mediated by DAF. Its reduction in APS points towards an increased vulnerability to complement mediated cell damage during trophoblast invasion and placenta formation. DAF therefore may exert a protective role during implantation and early pregnancy. PROK1 expression may be expected to show a reduction in luteal phase defect and APS and this warrents further investigation.

FGF2 is an angiogenic factor, which, by its endometrial localisation especially in and/or around blood vessels is thought to contribute to endometrial angiogenesis (Moller *et al*, 2001). This may be of importance in early pregnancy when PROK1 expression is elevated and the maternal vessels are undergoing remodelling as FGF-2 is also suggested to be involved in endothelial repair which is required after breach of the maternal vessels during trophoblast invasion (Wang DI *et al*, 1999). PROK1 may therefore play a role in endothelial repair during trophoblast invasion. FGF-2 is highly expressed in the mesometrial decidua at a time when extensive angiogenesis takes place in this tissue (Srivastava *et al*, 1998). However, other studies have suggested that the main expression of FGF-2 is located anti-mesometrially (Paria *et al*, 2001).

Expression of IL-8 has been localised to the peri-vascular cells of the secretory endometrium (Milne, 1999 *et al*, Jones, 1997 *et al*, Critchley, 1994 *et al*, Arici *et*

al, 1998). Localisation to the perivascular cells may imply a role for endothelial cell support.

As indicated above, COX-2 (cyclooxygenase-2) has been demonstrated by the knockout mouse model for COX-2 to have an essential role in implantation and decidualisation (Cheng and Stewart, 2003, Lim *et al*, 1997). COX-2 null mice are suggested to display impaired uterine angiogenesis at the implantation site due to a deficiency in prostaglandins (Matsumoto *et al*, 2002, Wang & Dey, 2006).

These data indicate PROK1 induces genes, which may contribute to adhesion during implantation, cell migration and invasion, repair and protection against toxic stimuli, blood vessel function as well as inhibitors of invasion. The action of these molecules *in vivo* may not depend just on induction by PROK1, and there is extensive opportunity for cross talk between the PROK1 induced genes to elevate or inhibit expression of others, e.g. PROK1 elevates both uPA and its inhibitor. Action may depend on other activators or inhibitors present in the endometrial milieu at the time of implantation. Clearly this work is just the 'tip of the iceberg' in terms of the *in vitro* and *in vivo* investigations required to delineate the action and role of PROK1 during the window of implantation. However it is encouraging that a number of genes regulated by PROK1 have previously been demonstrated to be elevated during the window of implantation, to have a reproductive phenotype as demonstrated by their knockout mouse model, or to be regulated by hCG, one of the first embryonic products. These imply PROK1 may indeed play a role in implantation or early pregnancy. The PROKR1 null mouse did not have a reported reproductive phenotype (Matsumoto *et al*, 2006). However, any defect in the PROKR1 null mouse may be compensated by PROK1 binding to PROKR2, as the receptors are highly homologous and may initiate similar signalling (Lin DC *et al*, 2002) in the endometrium thus avoiding a reproductive phenotype. The reproductive system does not develop in PROKR2 null animals (Matsumoto *et al*, 2006) due to an absence of GnRH neurones and a lack of gonadotropic support. In order to investigate the effect of an absence of both receptors, it would be of interest to investigate a dual PROKR1 knockout/PROKR2 conditional knockout mouse model and examine the reproductive phenotype. It would also be of

interest to investigate the reproductive phenotype of a PROK1 knockout mouse model, however, at present there are no reports of a PROK1 knockout in the literature. Alternatively, in the absence of these mouse models it would be of interest to use receptor inhibitors *in vivo* in order to investigate potential reproductive phenotypes and provide further information regarding the role of PROK1 in reproduction.

Chapter 6 - Prokineticin regulation of COX-2 expression and prostaglandin synthesis in PROKR1 Ishikawa cells

6.1. Introduction

In Chapter 3 it was demonstrated that PROK1 was elevated during the secretory phase of the cycle and further elevated during early pregnancy. It was subsequently established that PROK1 regulates expression of COX-2. The COX enzymes, mediate prostanoid biosynthesis and comprise 2 distinct enzymes, COX-1 and COX-2, which share 60% amino acid identity, and a variant form of COX-1 known as COX-3, which is produced by alternate splicing of COX-1 and retention of an intron (Chandrasekharan *et al*, 2002). The action of the COX-enzymes is the metabolism of arachidonic acid to produce prostaglandins. A role for the prostaglandins in female reproductive function has long been recognised. In 1967, Pickles described the prostaglandins in the human endometrium (Pickles, 1967). The prostaglandins, prostaglandin receptors, prostaglandin transporter and COX-2 expression have since been described in the pregnant and non-pregnant endometrium and uterine pathologies (Hofmann *et al*, 1983, Jones *et al*, 1997, Tong *et al*, 2000, Jabbour *et al*, 2001, Milne *et al*, 2003, Sales *et al*, 2004, Battersby *et al*, 2004b, Milne *et al*, 2001, Ferrandina *et al*, 2002, Smith *et al*, 1981a,1981b Ota *et al*, 2001, Milling-Smith *et al*, 2007).

COX-2 derived prostaglandins are important in a number of female reproductive functions. $PGF_{2\alpha}$, particularly, is important in ovulation for the extrusion of the ovum from the ovarian follicle (Plunkett *et al*, 1975). Prostaglandins are also important in parturition (Friel *et al*, 2005, Olson, 2003), during which time COX-2 expression is massively elevated above COX-1 expression in term human amnion (Slater *et al*, 1994).

There is an increasing amount of evidence pointing towards a role for COX-2 in implantation and early pregnancy. The COX-2 knockout mouse model provides compelling evidence for the importance of COX-2 in a number of female reproductive functions. The COX-2 null mice exhibit a reduced rate of ovulation and failure of fertilisation when compared with wild type mice. Indeed the eggs from COX-2 null mice were found to be developmentally abnormal (Lim *et al*,

1997). In contrast with the paper of Lim *et al* (1997), which reported a failure of wild type blastocyst to implant in the uteri of COX-2 null mice, Cheng & Stewart (2003) reported that pseudopregnant COX-2 null mice did display recognisable implantation on days 7 - 10 of pregnancy, which progressed to term. However, it was observed that the decidual weights were lower, exhibiting a delay in growth of about 24 hours when compared with wild type mice, and the pups were delivered about 1 day later in the COX-2 null than the wild type animals. In support of the requirement for COX-2 derived prostaglandins in decidualization was the observation that, in contrast to wild type mice, the COX-2 null mouse displays no decidualisation response to an intraluminal oil infusion on day 4 of pseudopregnancy (Lim *et al*, 1997). Indeed, PGE_2 has been demonstrated to induce decidualisation in human stromal cells (Frank, 1994, Dimitriadis, 2005) and administration of PGI_2 partially restores the decidualisation defect in COX-2 null mice (Lim *et al*, 1997).

In chapter 3 it was demonstrated that PROK1 is elevated in the mid-secretory phase of the menstrual cycle and further elevated in the pregnant endometrium. In chapter 5 it was discovered that COX-2 is a target gene for PROK1. Regulation of COX-2 by PROK1 may provide an important mechanism for prostaglandin production during early pregnancy. This study was therefore designed to investigate the role of PROK1 in inducing COX-2 expression and the biosynthesis of prostaglandins in the PROKR1 Ishikawa cell line model.

6.2. Materials and methods

6.2.1. Immunofluorescent histochemistry and confocal microscopy

Co-localization of PROKR1 with COX-2 was performed by dual immunofluorescence immunohistochemistry on endometrial tissue or first trimester decidua. Sections (5μM) were dewaxed in xylene and rehydrated using decreasing concentrations of ethanol. Antigen retrieval was performed for 5 minutes in a pressure cooker in boiling 0.1% citrate buffer (pH 6.0). Endogenous peroxidase activity was quenched with 3% (vol/vol) H_2O_2 in methanol at room temperature. Normal horse serum block (5% serum in PBS with 0.05% BSA) was applied for one hour before overnight incubation at 4°C

with goat anti-COX-2 antibody at 1:50 or goat IgG for control sections. Sections were washed in PBS, COX-2 treated sections were incubated with biotinylated horse anti goat, followed by washing in PBS and incubation with the fluorochrome Streptavidin 488 diluted 1:200 in PBS. Sections were re-blocked with 5% normal goat serum block diluted in PBS and incubated with rabbit anti-human PROKR1 (1:500) overnight at 4°C. Control sections were incubated with rabbit IgG. Sections were washed in PBS and incubated with peroxidase goat anti-rabbit, 1:200 in PBS, followed by washing in PBS and incubation with the fluorochrome Cyanine 3 (red) at 1:50 in substrate provided with the kit. Sections were washed in PBS and then incubated with the nuclear counterstain ToPro diluted 1:2000 in PBS. Sections were mounted in Permafluor and fluorescent images were visualised using a laser-scanning confocal microscope (LSM 510, Carl Zeiss) with a 40 x 1.4 aperture oil immersion lens.

6.2.2. Cell culture and treatments

6.2.2.1. Time course analysis of PROK1 mediated COX-2 expression

In order to examine the temporal regulation of COX-2 by PROK1, PROKR1 or WT Ishikawa cells were treated with 40nM PROK1 or vehicle for 0, 2, 4, 6, 8, 12 or 24 hours. Cells were subsequently lysed in NP40 lysis buffer and processed for Western immunoblot analysis (as described in Section 6.2.4) or lysed in TRI reagent, followed by RNA extraction and processing for Taqman quantitative PCR analysis (as described in Section 6.2.3).

6.2.2.2. Mechanism of PROK1 mediated COX-2 RNA and protein expression

In order to examine the signalling pathway activation leading to PROK1 mediated COX-2 expression, PROKR1 Ishikawa cells were preincubated with chemical inhibitors of Gq protein (YM254890), PLC-β (U73122), Ca^{2+} (calcium chelators, BAPTA-AM), cSrc (PP2), EGFR kinase (AG1478) or MEK (PD98059; working concentrations for inhibitors indicated in Table 14). In order to examine PROK1 mediated COX-2 RNA or protein

expression, PROKR1 Ishikawa cells were subsequently treated with vehicle or 40nM PROK1 for 6 hours or 8 hours respectively.

In order to determine if PROK1 mediated COX-2 expression was transcriptionally activated, PROKR1 Ishikawa cells were pre-incubated with an inhibitor of transcriptional activation (Actinomycin D). PROKR1 Ishikawa cells were subsequently treated with vehicle or 40nM PROK1 for 4, 6 or 8 hours.

Cells were lysed in NP40 lysis buffer and processed for Western immunoblot analysis (as described in Section 6.2.4), or lysed in TRI reagent, followed by RNA extraction and processing for Taqman quantitative PCR analysis (as described in Section 6.2.3).

Table 14. Inhibitor concentrations

Inhibitor	Molecule Inhibited	Inhibitor concentration
YM254890	Gq protein	1μM
U73122	Phospholipase C	10μM
BAPTA-AM	Calcium	50μM
PP2	c-Src	10μM
AG1478	EGFR	200nM
PD98059	MEK	50μM
Actinomycin D	Transcriptional activation	250ng/ml

6.2.3. Polymerase chain reaction

6.2.3.1.RNA extraction

RNA was extracted using TRI-reagent and the phenol-chloroform method of RNA extraction as described in section 2.5. Briefly, cells were lysed in TRI-reagent and a homogenous lysate achieved by mixing by pipetting. Lysed samples were then loaded into heavy gel phase lock tubes with he addition of bromo-chloro propane. Tubes were shaken vigorously to ensure adequate mixing of the two solutions. Samples were then processed as described in section 2.5.

6.2.3.2.Taqman quantitative PCR

Expression of RNA within cultured cells was examined by Taqman quantitative PCR analysis as described in section 2.5. Briefly, cDNA was prepared in a random hexamer primed reaction using 200ng RNA per reaction. Quantitative PCR analysis was performed on the cDNA using specific primer probe combinations to detect COX-2 (sequence given in Table 5), these were designed in house and custom synthesised. The probe was given a FAM fluorescent label with a TAMRA quencher. Gene expression was normalized by including Vic labelled primer-probe mix to detect the 18s ribosomal subunit (sequence given in Table 5) as a loading control for the amount of cDNA added in each sample.

Reaction mixes were loaded in duplicate onto a 96-well MicroAmp fast optical reaction plate for analysis on an ABI7900 HT Fast Real-Time PCR machine. Data were analysed and processed using sequence detector version 1.6.3. Results were expressed as relative to a positive RNA standard included in all reactions. The data were analysed using the comparative C_T method for relative quantification.

6.2.4. Western immunoblot analysis

After lysis of PROKR1 Ishikawa cells in NP40 lysis buffer, protein concentration of lysates was quantified by modified Bradford protein assay kits as described in section 2.7. In order to examine COX-2 protein expression 40μg of protein was resuspended in Laemmli buffer (Section 2.12) and boiled for 5 minutes to denature proteins. Proteins were resolved on a 4 - 20% SDS-PAGE gradient gel and subsequently immunoblotted onto a PVDF membrane. Non-specific antibody binding was prevented by incubation of the immunoblot with low fluorescence blocking buffer for one hour (LiCor). Immunoblots were subsequently exposed to specific antibodies for COX-2 (goat anti COX-2, 1:500) and the loading control β-actin (goat anti β-actin, 1:800) overnight at 4°C with gentle motion. The immunoblots were washed and exposed to a fluorescently labelled antibody directed against goat immunoglobulin. Proteins were visualised using a LiCor fluorescent scanner and densitometry analysis performed using LiCor software. Immunoblots were quantified by dividing the

value obtained for COX-2 by the value obtained for β-actin and expressing these as fold above vehicle treated control.

6.2.5. Transient transfection of COX-2 promoter reporter luciferase constructs and dominant negative isoforms of signalling molecules

6.2.5.1. Transient transfection of COX-2 luciferase promoter reporter constructs

In order to examine the transcriptional regulation of COX-2 by PROK1 the full length COX-2 luciferase promoter reporter construct was utilized (described in Section 2.3). PROKR1 Ishikawa cells were seeded at a density of 100,000 cells/well in a 12-well plate. 1.5 μg of the COX-2 promoter reporter construct linked to firefly luciferase (described in section 2.3) was co-transfected with 0.15μg of internal control pRL-TK into each well of PROKR1 Ishikawa cells for 6 hours in the presence of Superfect transfection reagent. The transfection mixture was removed and cells incubated overnight in complete DMEM Glutamax cell culture media.

In order to investigate the temporal regulation of PROK1 mediated COX-2 promoter activation, PROKR1 Ishikawa cells were subjected to serum starvation overnight followed by stimulation with 40nM PROK1 for 2, 4, 6 or 8 hours.

In order to investigate the signaling pathways involved in PROK1 mediated activation of the COX-2 promoter, the transfected cells were pre-incubated with inhibitors of signaling molecules for one hour (concentrations given in table 14) prior to stimulation with 40nM PROK1 for 6 hours.

Reactions were terminated by removal of agonist and the cells were washed in ice-cold PBS. The cells were lysed in passive lysis buffer and processed for COX-2 luciferase assay as described in Section 6.2.6.

6.2.5.2.Co-transfection of COX-2 luciferase promoter reporter constructs and dominant negative isoforms of signalling molecules

In order to examine the role of signalling molecules in PROK1 mediated COX-2 promoter activation, PROKR1 Ishikawa cells were co-transfected with the COX-2 luciferase promoter reporter construct and dominant negative isoforms of signaling molecules. 1.5 μg of the COX-2 promoter reporter construct linked to firefly luciferase (described in section 2.3) was co-transfected with 1.5 μg of empty vector (pcDNA3) or DN-cSrc, DN-EGFR, DN-Ras or DN-MEK with 0.15μg of internal control pRL-TK into PROKR1 Ishikawa cells for 6 hours in the presence of Superfect transfection reagent. The transfection mixture was removed and cells incubated overnight in complete DMEM Glutamax cell culture media. PROKR1 Ishikawa cells were subjected to serum starvation overnight followed by stimulation with 40nM PROK1 for 6 hours. Reactions were terminated by removal of agonist and the cells were washed in ice-cold PBS. The cells were lysed in passive lysis buffer and processed for COX-2 luciferase assay as described in Section 6.2.6.

6.2.6. COX-2 luciferase assay

The activity of both COX-2 promoter firefly luciferase and renilla within cell lysates was determined using the dual luciferase assay kit (Promega). Total luciferase activity was determined by dividing the relative light units generated by the firefly luciferase by relative light units generated by the renilla luciferase in the same reaction. Fold increase in luciferase activity was calculated by dividing the total luciferase activity in cells treated with PROK1 by the total luciferase activity in cells treated with vehicle or transfected with empty vector.

6.2.7. Prostaglandin ELISA

PGE_2 and $PGF_{2\alpha}$ concentration in cell culture media following treatment with 40nM PROK1 was determined by ELISA. This has been described fully in section 2.10.2. Briefly, PROKR1 Ishikawa cells were stimulated with 40nM PROK1 alone or in the presence of 10μM of the specific COX-2 inhibitor NS-398 for 0, 2, 4, 6, 8, 12 or 24 hours. Arachidonic acid was added to a final concentration of 3μg/ml 4 hours prior to the time point termination. For assay

of $PGF_{2\alpha}$ the media was removed and frozen at -20°C prior to assay. In order to stabilise PGE_2 an equal volume of MOX was added to the culture medium before storage at -20°C. Prostaglandin concentrations were subsequently assayed by ELISA as described in section 2.10.

6.2.8. Statistics

Statistical analysis of the data in this study was carried out by ANOVA with Fishers PLSD (predicted least squares difference) applied, using Statview 5.0 (Abacus Concepts). The data is presented as mean ± SEM of at least 3 experiments.

6.3. Results

6.3.1. Co-expression of PROKR1 and COX-2 in human endometrium and first trimester decidua

As indicated in chapter 5, global gene array analysis suggests that expression of COX-2 is under regulatory control of PROK1 via PROKR1. In order to investigate the potential physiological regulation of COX-2 expression by PROK1 *in vivo*, tissue localisation of COX-2 and PROK1 were examined by fluorescent immunohistochemistry and confocal microscopy. Mid-secretory phase endometrium and first trimester decidua tissue were examined for the expression of PROKR1 and COX-2. Expression of COX-2 (red channel) and PROKR1 (green channel) localized to the glandular epithelial cells and some stromal cells of mid secretory endometrium and first trimester decidua (yellow channel, Figure 6.1). The co-localisation of PROKR1 and COX-2 in the same cellular compartment suggests potential regulation of COX-2 by PROK1-PROKR1 interaction.

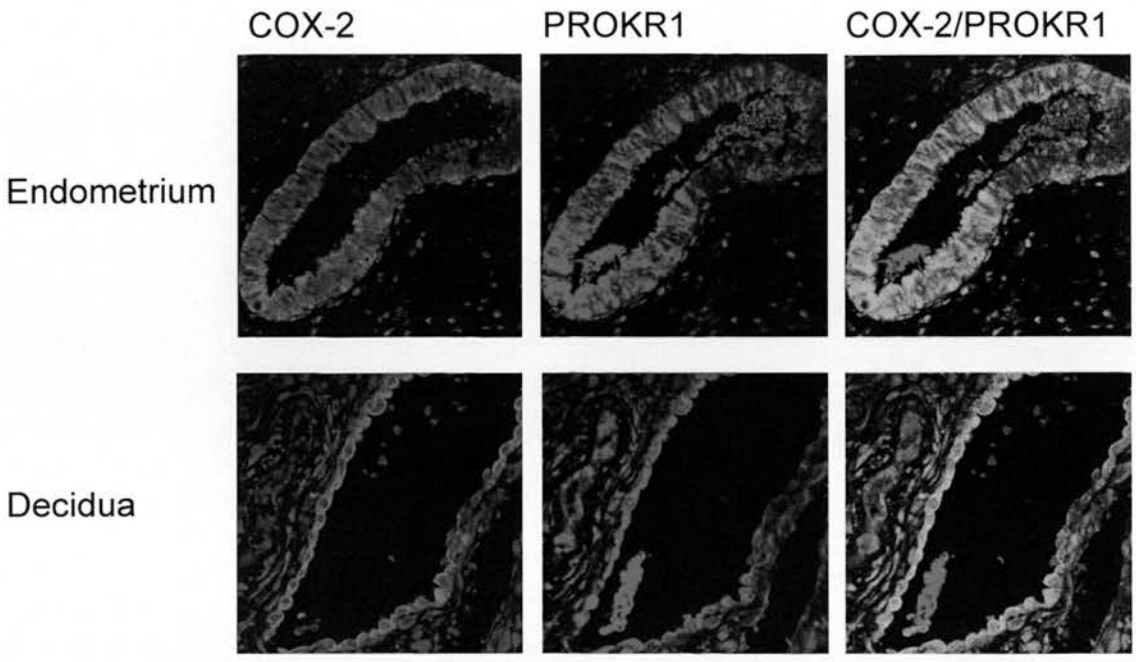


Figure 6.1. Co-localization of PROKR1 and COX-2 in endometrium and first trimester decidua. Examination of PROKR1 (red channel) and COX-2 (green channel) expression in mid-secretory endometrium and first trimester decidua by fluorescent immunohistochemistry and confocal microscopy confirmed co-localisation of PROKR1 and COX-2 (yellow channel) in glandular epithelial cells and some stromal cells.

6.3.2. Temporal regulation of PROK1 mediated COX-2 expression

6.3.2.1. Temporal regulation of PROK1 induced COX-2 RNA expression in PROKR1 Ishikawa cells

PROK1-PROKR1 mediated regulation of COX-2 expression has been demonstrated at 8 hours by global gene array analysis and validation of expression at 8 hours (Chapter 5). In order to examine the temporal regulation of PROK1 mediated COX-2 expression, WT and PROKR1 Ishikawa cells were treated with 40nM PROK1 for 0, 2, 4, 6, 8, 12 and 24 hours followed by Taqman quantitative PCR analysis for COX-2 mRNA expression. PROK1 induced expression of COX-2 in PROKR1 Ishikawa cells was rapid with elevation in expression evident at 2 hours. PROK1 induced COX-2 expression was maximal at 6 hours (6.9 ± 1.2 fold above vehicle treated control, $p<0.01$, Figure 6.2), which had declined to basal levels by 24 hours. No elevation of COX-2 expression was revealed at any time in WT Ishikawa cells treated with 40nM PROK1. This demonstrates

that PROK1 induced expression of COX-2 mRNA is rapid with elevation at 2 hours after treatment with 40nM PROK1 that is sustained for up to 8 hours.

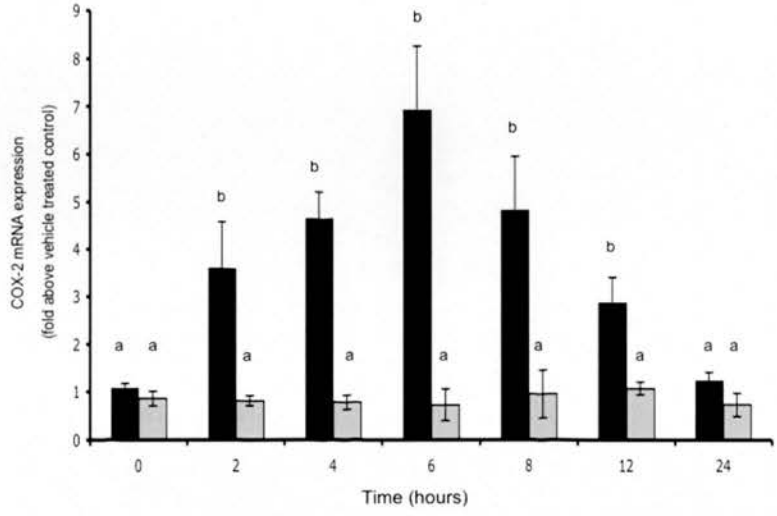


Figure 6.2. Temporal regulation of PROK1 mediated COX-2 RNA expression. PROKR1 and WT Ishikawa cells were stimulated with 40nM PROK1 for 0, 2, 4, 6, 8, 12 and 24 hours. COX-2 expression was assessed by Taqman quantitative PCR analysis. COX-2 expression was normalized for loading against expression of 18s and relative to a single endometrium control. Data are expressed as fold above vehicle treated control. WT Ishikawa cells are represented by grey bars, PROKR1 Ishikawa cells are represented by black bars. COX-2 expression is rapidly elevated by PROK1 in PROKR1 Ishikawa cells and is maximal at 6 hours. No elevation in COX-2 expression is evident in WT Ishikawa cells at any time point examined. Data are presented as mean ± SEM. (b is significantly different from a $p<0.01$). **NB**. This figure was presented as part of composite figure 5.2 and has been reproduced here for clarity and interpretation of relevant results within this section.

6.3.2.2. Temporal regulation of COX-2 protein expression in PROKR1 Ishikawa cells

Subsequently, the temporal regulation of PROK1 induced COX-2 protein expression was investigated. PROKR1 Ishikawa cells were treated with vehicle or 40nM PROKR1 for 2, 4, 6, 8, 12 and 24 hours and COX-2 expression determined by Western immunoblot analysis. COX-2 protein expression was rapid with some expression evident at 2 - 4 hours. COX-2 protein expression peaked at 8 hours (9.59 ± 2.1 fold above vehicle treated control, $p<0.01$, Figure 6.3) and had declined to basal levels by 24 hours.

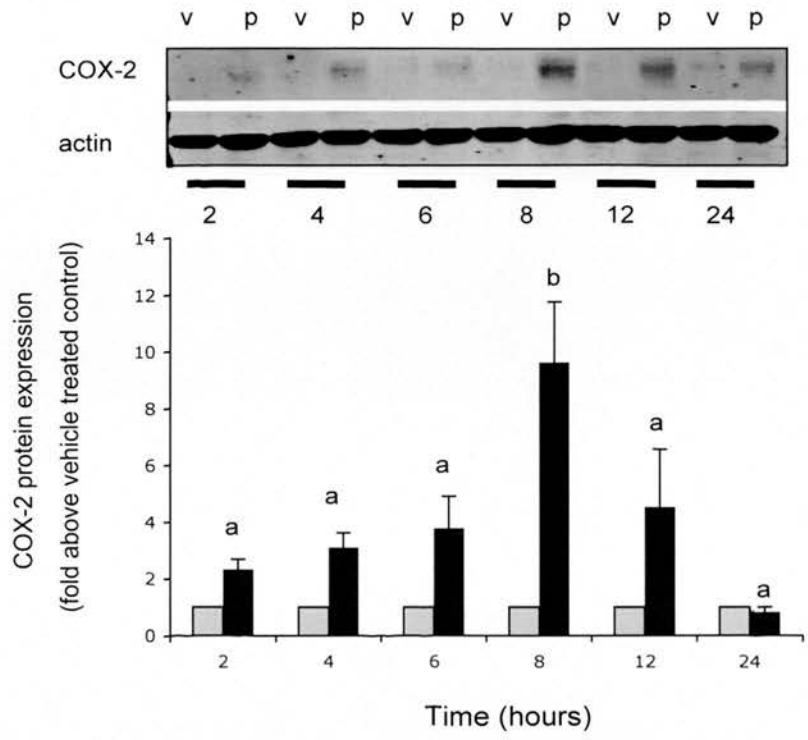


Figure 6.3. Temporal regulation of PROK1 induced COX-2 protein expression. PROKR1 Ishikawa cells were treated with 40nM PROK1 for 2, 4, 6, 8, 12 and 24 hours. Proteins were extracted and 40μg subjected to Western immunoblot analysis for COX-2 expression (COX-2). The total amount of protein in cell lysates was determined by probing the same blot with antibodies recognising β-actin (actin). COX-2 expression was normalized for loading against expression of actin. Data are expressed as fold above vehicle treated control. Grey bars represent vehicle treated cells, v, black bars represent PROK1 treated cells, p. PROK1 mediated COX-2 protein expression is maximal at 8 hours in response to 40nM PROK1 in PROKR1 Ishikawa cells. A representative Western immunoblot is shown with semi-quantitative densitometric analysis. Data are presented as mean ± SEM. (b is significantly different from a $p<0.01$).

6.3.3. Mechanism of PROK1 mediated COX-2 expression

6.3.3.1. PROK1 mediated COX-2 expression is partially transcriptionally regulated

There is evidence to suggest that COX-2 expression may be only partially transcriptionally regulated. A report in 2000 revealed that activation of Gq coupled P2Y nucleotide receptors induced approximately 137 fold elevation in rat COX-2 expression by one hour.

However, transcriptional induction only accounted for about 3-fold of this value (Xu *et al*, 2000). The paper provided strong evidence for partial transcriptional regulation and partial transcription stabilisation. This may be the case for COX-2 expression in other systems. In order to investigate PROK1 induced transcriptional regulation of COX-2 expression, Actinomycin D, a powerful inhibitor of transcriptional activity, was used. PROKR1 Ishikawa cells were treated with 40nM PROK1 for 4, 6 and 8 hours in the presence or absence of 250ng/ml Actinomycin D. PROK1 induced expression of COX-2 was maximal at 6 hours (Figure 6.4). At this time point, treatment with Actinomycin D reduced PROK1 induced COX-2 expression to about 50% of its maximal value (Figure 6.4, $p<0.05$). These data suggest that PROK1 induced expression of COX-2 is partially under transcriptional control and partially under the control of transcriptional stabilisation mechanisms.

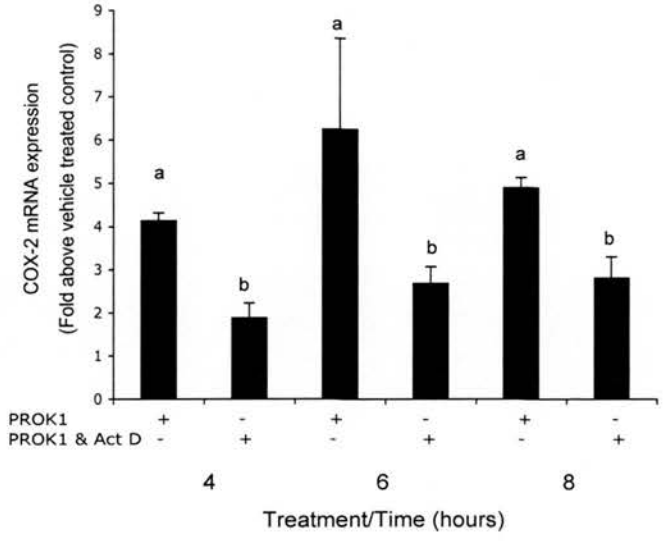


Figure 6.4. Inhibition of PROK1 induced transcriptional activation of COX-2. PROKR1 Ishikawa cells were treated with 40nM PROK1 alone or in the presence of Actinomycin D for 4, 6, and 8 hours. COX-2 expression was assessed by Taqman quantitative PCR analysis. COX-2 expression was normalized for loading against expression of 18s and relative to a single endometrial control. Data are expressed as fold above basal levels. Upon treatment of PROKR1 Ishikawa cells with 40nM PROK1, COX-2 expression is maximal at 6 hours. This is inhibited to about 50% of the maximal value by Actinomycin D suggesting that PROK1 induced COX-2 expression is partially transcriptionally activated. + denotes presence of agent, - denotes absence of agent. Data presented as mean ± SEM (b is significantly different from a $p<0.05$).

6.3.3.2. Mechanism of PROK1 mediated transcriptional activation

In order to investigate PROK1 induced transcriptional up-regulation of COX-2, PROKR1 Ishikawa cells were transfected with the full-length COX-2 luciferase promoter reporter construct. Initially the effect of PROK1 on the temporal regulation of COX-2 transcriptional activation was investigated. PROKR1 Ishikawa cells, transfected with the COX-2 luciferase promoter reporter construct, were treated with 40nM PROK1 for 0, 2, 4, 6 and 8 hours. Luciferase assay revealed PROK1 induced activity driven by the COX-2 promoter, to be maximal at 6 - 8 hours (Figure 6.5 A, COX-2 promoter, $p<0.05$). Therefore all subsequent work examining PROK1 induced transcriptional activation was performed at 6 hours. There is a risk of non-specific luciferase promoter activity when the PGL3 vector is used due to the structure of this vector. In order to rule out non-specific activation, PROKR1 Ishikawa cells were transfected with the empty PGL3 vector and treated with 40nM PROK1 for 0, 2, 4, 6 and 8 hours. Treatment of empty vector transfected PROKR1 cells with 40nM PROK1 revealed no increase in luciferase activity over vehicle treated controls at any time. These data suggest the results would not be confounded by inaccuracies arising from non-specific stimulation (Figure 6.5 A, PGL3 basic).

In order to investigate the signalling pathway regulating PROK1 mediated COX-2 promoter activation, PROKR1 Ishikawa cells were transfected with the full length COX-2 luciferase promoter reporter construct and treated with 40nM PROK1 alone or in the presence of chemical inhibitors of Gq protein, PLC-β, Ca^{2+}(calcium chelator), cSrc, EGFR and MEK for 6 hours. 40nM PROK1 induced a 2.05±0.11 fold increase in COX-2 transcriptional activity (Figure 6.5 B, $p<0.01$). This was inhibited following co-treatment with PROK1 and Gq inhibitor (Figure 6.5 B, lane 3), PLC-β inhibitor (Figure 6.5 B, lane 4) Ca^{2+} inhibitor (Figure 6.5 B, lane 5), cSrc inhibitor (Figure 6.5 B, lane 6) EGFR inhibitor (Figure 6.5 B, lane 7) and MEK inhibitor (Figure 6.5 B, lane 8).

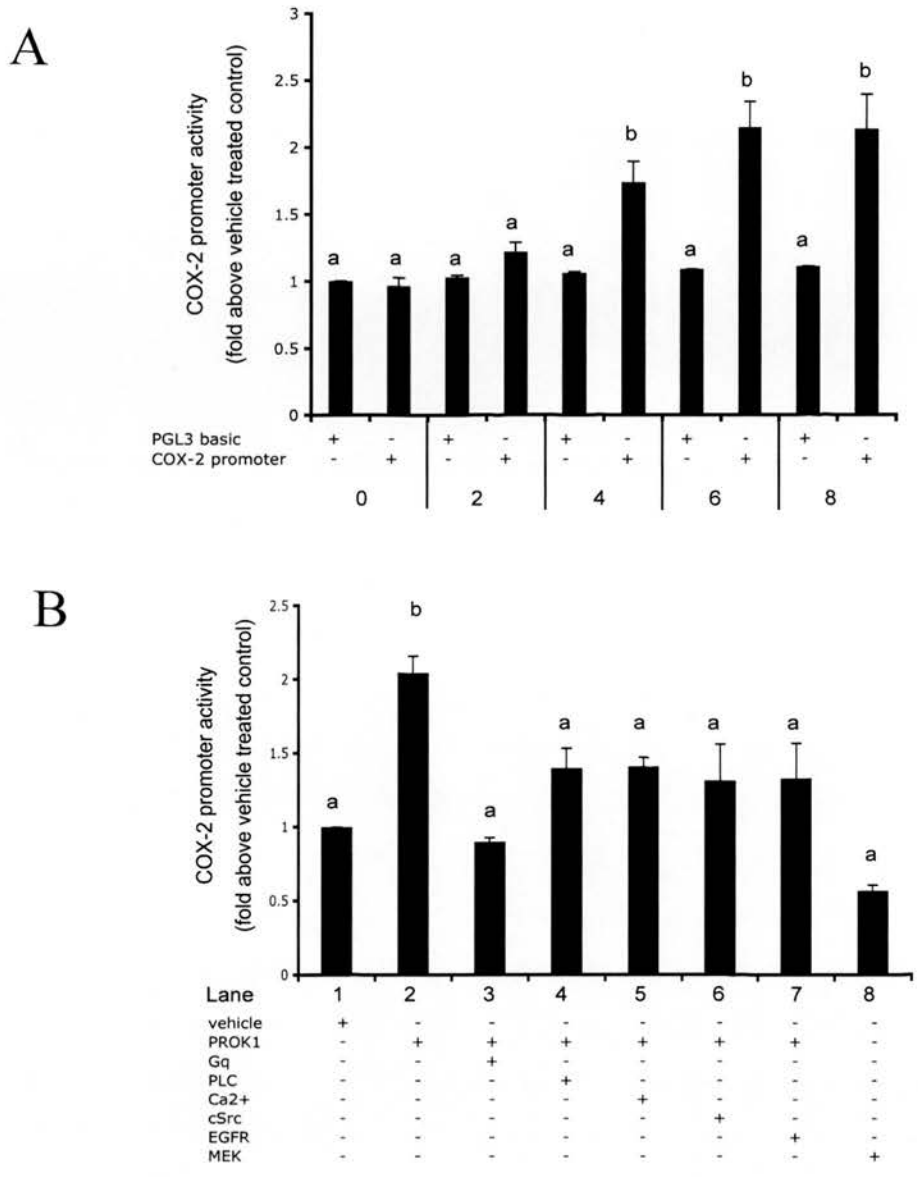


Figure 6.5. PROK1 mediated transcriptional activation of COX-2. A. PROKR1 Ishikawa cells were co-transfected with the luciferase vector PGL3 basic or the full length COX-2 promoter firefly luciferase reporter with internal control pRL-TK (renilla). Transfected PROKR1 Ishikawa cells were stimulated with 40nM PROK1 for 0, 2, 4, 6 and 8 hours. COX-2 promoter activation was assessed by luciferase assay analysis. B. PROKR1 Ishikawa cells were co-transfected with full-length COX-2 promoter firefly luciferase reporter and internal control pRL-TK (renilla). Transfected cells were stimulated with 40nM PROK1 alone or in the presence of inhibitors of Gq protein (YM254890), PLC-β (U73122), Ca^{2+} (BAPTA-AM), cSrc (PP2), EGFR (AG1478) or MEK (PD98059) for 6 hours. COX-2 promoter activation was assessed by luciferase assay analysis. COX-2 luciferase activity was normalised against renilla luciferase activity. Data are presented as fold above vehicle treated control. COX-2 promoter activation is elevated at 4 hours in PROKR1 Ishikawa cells transfected with the full length COX-2 promoter reporter

luciferase construct, and peaks at 6 - 8 hours. Co-treatment of PROKR1 Ishikawa cells transfected with the full length COX-2 promoter reporter luciferase construct with 40nM PROK1 and chemical inhibitors significantly inhibited maximal PROK1 induced COX-2 promoter activation at 6 hours. + denotes presence of agent, - denotes absence of agent. Data are presented as mean ± SEM (b is significantly different from a $p<0.05$).

6.3.3.3. Effect of dominant negative isoforms of signalling molecules on PROK1 mediated COX-2 transcriptional activation

In order to confirm the role suggested by the use of chemical inhibitors of signalling molecules on PROK1 mediated transcriptional activation of COX-2, dominant negative constructs of cSrc, EGFR, MEK and the small monomeric G-protein Ras were employed. PROKR1 Ishikawa cells were co-transfected with the full length COX-2 promoter and empty vector or dominant negative cDNA encoding cSrc, EGFR, Ras and MEK mutants. Transfected PROKR1 Ishikawa cell were then stimulated with 40nM PROK1 for 6 hours inducing a 1.78±0.19 fold increase in COX-2 transcriptional activity (Figure 6.6, lane 2, $p<0.01$) in empty vector transfected cells. PROK1 mediated elevation in COX-2 transcriptional activity at 6 hours was reduced to almost basal levels by co-expression of dominant negative constructs encoding mutants of cSrc (Figure 6.6, lane 4), EGFR (Figure 6.6, lane 5), Ras (Figure 6.6, lane 6) and MEK (Figure 6.6, lane 7). This further implicates the activation of cSrc, EGFR, Ras, MEK by PROK1 in the transcriptional activation of COX-2.

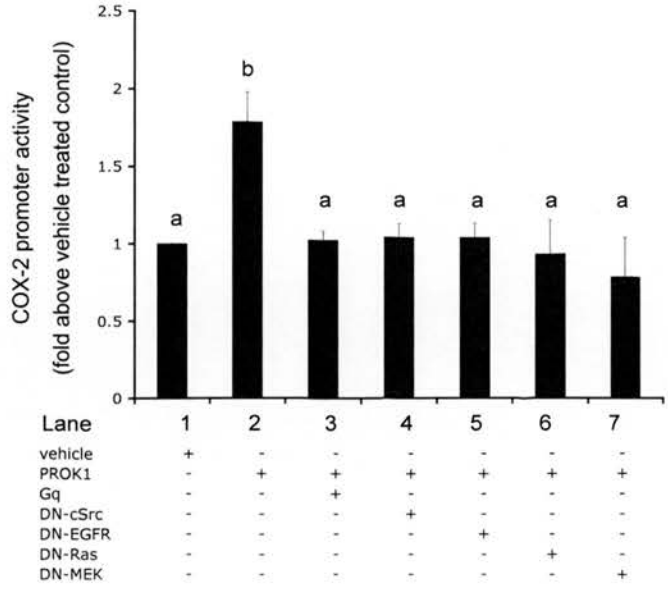


Figure 6.6. PROK1 mediated transcriptional activation of COX-2 is dependent on activation of multiple signalling molecules. PROKR1 Ishikawa cells were co-transfected with full-length COX-2 promoter firefly luciferase reporter along with dominant negative cDNA constructs encoding mutants of cSrc, EGFR, Ras or MEK and internal control pRL-TK (renilla). Transfected cells were stimulated with 40nM PROK1 for 6 hours. COX-2 promoter activation was assessed by luciferase activity analysis. COX-2 luciferase activity was normalised against renilla luciferase activity. Data are expressed as fold above vehicle treated control. Expression of dominant negative isoforms of signalling molecules in PROKR1 Ishikawa cells transfected with the full length COX-2 promoter reporter luciferase construct significantly inhibited the maximal PROK1 induced COX-2 promoter activation at 6 hours. + denotes presence of agent, - denotes absence of agent. Data are presented as mean ± SEM (b is significantly different from a $p<0.01$).

6.3.3.4. Mechanism of PROK1 mediated COX-2 RNA expression

Transcriptional activity of COX-2 has been demonstrated as dependent on activation of multiple signalling molecules. In order to examine how this affected RNA expression, PROKR1 Ishikawa cells were treated with 40nM PROK1 alone or in the presence of inhibitors of Gq protein, PLC-β, Ca^{2+} (calcium chelator), cSrc, EGFR and MEK. Treatment of PROKR1 Ishikawa cells with 40nM PROK1 for 6 hours induced an 11.76±1.93 fold increase in COX-2 RNA expression (Figure 6.7, lane 2, $p<0.01$), which was reduced by treatment with inhibitors of PLC-β (Figure 6.7, lane 4), Ca^{2+}(Figure 6.7, lane 5), cSrc (Figure 6.7, lane 6), EGFR (Figure 6.7, lane 7) and MEK (Figure 6.7, lane 8). However,

treatment with the Gq protein inhibitor reduced COX-2 expression to basal levels (Figure 6.7, lane 8).

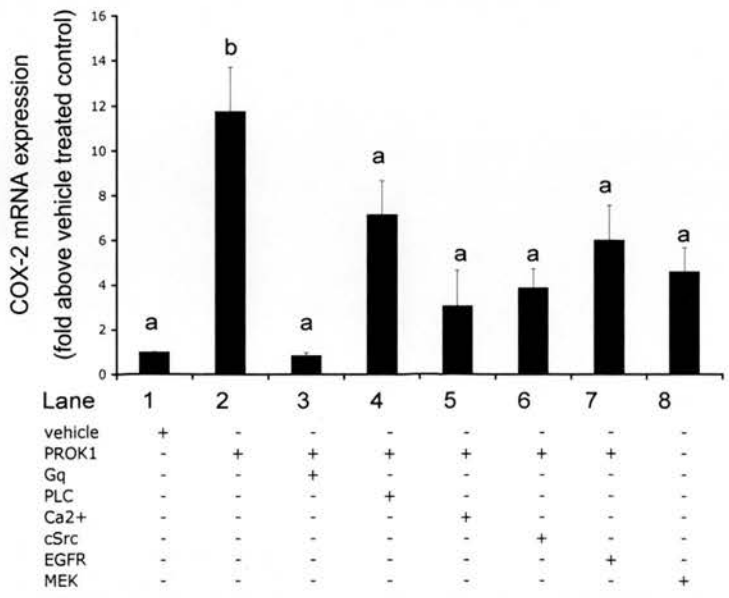


Figure 6.7. PROK1 mediated COX-2 RNA expression is dependent on activation of multiple signalling molecules. PROKR1 Ishikawa cells were treated with 40nM PROK1 in the presence or absence of chemical inhibitors of Gq protein (YM254890), PLC-β (U73122), Ca^{2+} (calcium chelator BAPTA-AM), cSrc (PP2), EGFR (AG1478) or MEK (PD98059) for 6 hours. COX-2 expression was assessed by Taqman quantitative PCR analysis. COX-2 expression was normalized for loading against expression of 18s and relative to an internal endometrial control. Data are expressed as fold above vehicle treated control. Co-treatment with 40nM PROK1 and chemical inhibitors significantly inhibited the maximal PROK1 induced COX-2 expression at 6 hours. + denotes presence of agent, - denotes absence of agent. Data are presented as mean ± SEM. (b is significantly different from a $p<0.01$).

6.3.3.5. Mechanism of PROK1 mediated COX-2 protein expression.

In order to examine the impact of signalling molecule activation on PROK1 induced COX-2 protein expression, PROKR1 Ishikawa cells were treated with 40nM PROK1 for 8 hours alone or in the presence of inhibitors of Gq protein, PLC-β, Ca^{2+} (calcium chelator), cSrc, EGFR and MEK. Treatment of PROKR1 Ishikawa cells with 40nM PROK1 for 8 hours induced an 11.65 ± 2.04 fold increase in COX-2 protein expression ($p<0.001$, Figure 6.8, lane 2). PROK1 mediated COX-2

protein expression was significantly reduced by co-treatment of PROKR1 Ishikawa cells with PROK1 and inhibitors of Gq protein (Figure 6.8, lane 3), PLC-β (Figure 6.8, lane 4), Ca^{2+} (Figure 6.8, lane 5), cSrc (Figure 6.8, lane 6), EGFR (Figure 6.8, lane 7) and MEK (Figure 6.8, lane 8). These data correspond with that obtained from investigation of the role of inhibitors of signalling molecules on PROK1 mediated COX-2 RNA expression.

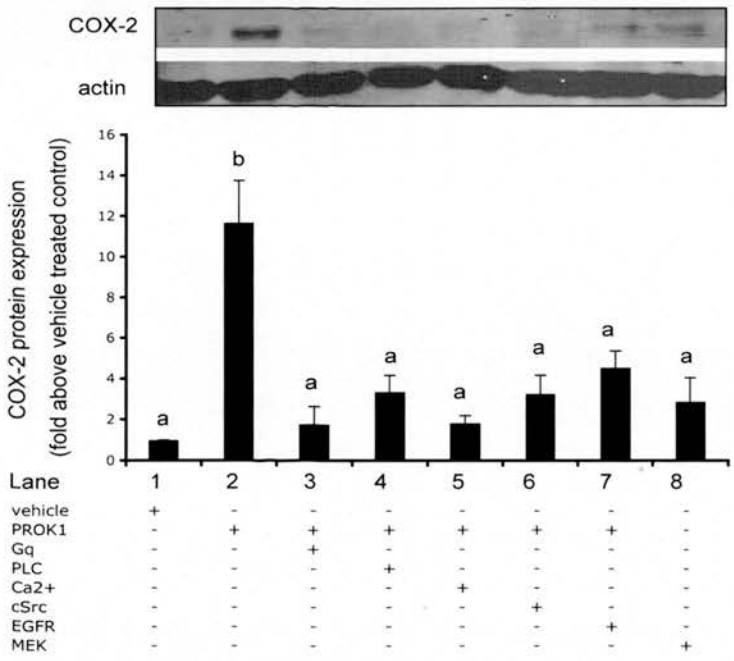


Figure 6.8. PROK1 mediated COX-2 protein expression is dependent on activation of multiple signalling molecules. PROKR1 Ishikawa cells were treated with 40nM PROK1 in the presence or absence of chemical inhibitors of Gq protein (YM254890), PLC-β (U73122), Ca^{2+} (calcium chelator BAPTA-AM), cSrc (PP2), EGFR (AG1478) or MEK (PD98059) for 8 hours. Proteins were extracted and 40μg subjected to Western immunoblot analysis for COX-2 (COX-2). The total protein content of cell lysates was determined by probing the same blot with antibodies recognising β-actin (actin). COX-2 expression was normalized for loading against expression of actin. Data are expressed as fold above vehicle treated controls. Co-treatment with 40nM PROK1 and chemical inhibitors significantly inhibited the maximal PROK1 induced COX-2 expression at 8 hours. A representative Western immunoblot is shown with semi-quantitative densitometric analysis. + denotes presence of agent, - denotes absence of agent. Data are presented as mean ± SEM. (b is significantly different from a $p<0.001$).

6.3.4. De novo synthesis of prostaglandins mediated by PROK1

COX-2 is the rate-limiting enzyme in production of prostaglandins. As PROK1 induces expression of COX-2 protein, it was therefore of interest to determine whether this resulted in prostaglandin synthesis. PROKR1 Ishikawa cells were stimulated with 40nM PROK1 for 0, 2, 4, 6, 8, 12 and 24 hours in the presence of 3μg/ml arachidonic acid in order to facilitate prostaglandin production. At the designated times, media was removed and assayed for PGE_2 and $PGF_{2\alpha}$ concentrations. PROK1 stimulated production of PGE_2 and $PGF_{2\alpha}$ in a time dependent manner with production of both prostaglandins initiated at 6 hours and reaching maximal concentrations by 12 hours (15.28 ± 2.9 and 5.29 ± 0.5 fold above vehicle treated control for PGE_2 and $PGF_{2\alpha}$ respectively, $p<0.001$, Figure 6.9 A and B).

6.3.4.1.PROK1 mediated prostaglandin synthesis is dependent on COX-2 expression

In order to investigate the role of PROK1 induced COX-2 expression in PROK1 mediated prostaglandin production, PROKR1 Ishikawa cells were treated with 40nM PROK1 in the absence or presence of the specific COX-2 inhibitor NS-398 for 0, 2, 4, 6, 8, 12 and 24 hours with the addition of 3μg/ml arachidonic acid in order to facilitate prostaglandin production. Co-treatment of PROKR1 Ishikawa cells with PROK1 and NS-398 significantly reduced PROK1 induced prostaglandin production at all time points examined. This suggests that PROK1 mediated de novo synthesis of prostaglandins is initiated via the expression of COX-2 (Figure 6.9 A and B for PGE_2 and $PGF_{2\alpha}$ respectively).

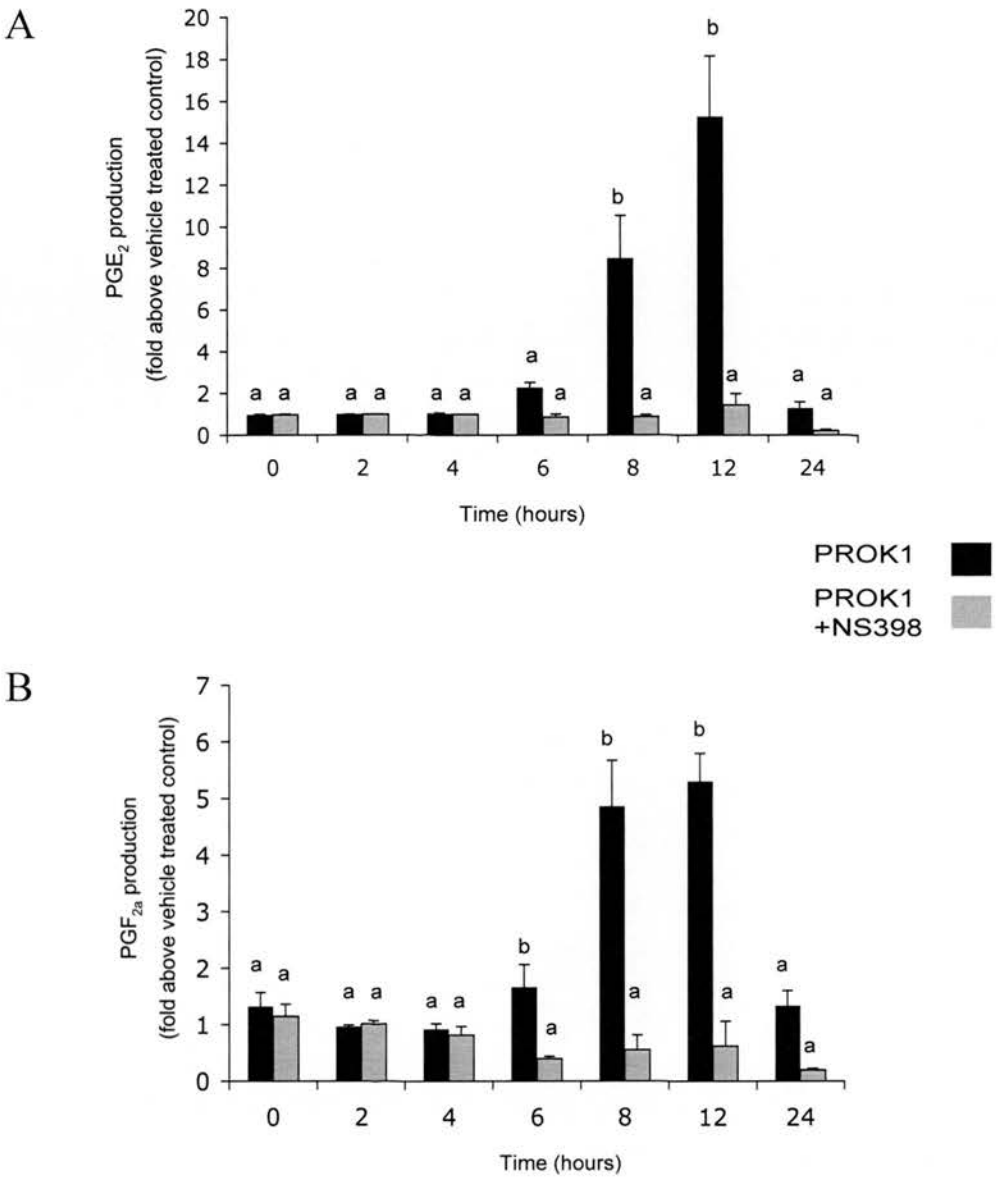


Figure 6.9. PROK1 induced de novo synthesis of prostaglandins in PROKR1 Ishikawa cells. PROKR1 Ishikawa cells were treated with 40nM PROK1 for 0, 2, 4, 6, 8, 12 and 24 hours in the presence or absence of specific COX-2 inhibitors with the addition of 3μg/ml arachidonic acid. Prostaglandin concentration in cell culture media was measured by ELISA. Data are expressed as fold above vehicle treated control. PROK1 mediated synthesis of PGE_2 (A) and $PGF_{2\alpha}$ (B) was maximal at 12 hours and was reduced to basal levels by co-treatment with NS-398 at all time points examined. Data are presented as mean ± SEM. (b is significantly different from a $p<0.001$).

6.4. Discussion

PROK1 is elevated during the secretory phase of the menstrual cycle with further elevation of both PROK1 and PROKR1 in the human early pregnancy endometrium. In this study the regulation of COX-2 expression by PROK1, suggested by gene array analysis of PROKR1 Ishikawa cells treated with 40nM PROK1 for 8 hours, was further confirmed. Expression of PROKR1 and COX-2 in secretory phase endometrium and pregnant endometrium (first trimester decidua) localize to the glandular epithelial cells and some cells of the stromal compartment. This may suggest a potential physiological regulation of COX-2 expression by PROK1 in these cellular compartments *in vivo*. COX-2 expression induced by PROK1 via PROKR1 is temporally regulated with elevation in RNA expression noted at 2 hours, and a peak in PROK1 induced COX-2 RNA expression at 6 hours. No elevation in COX-2 expression was noted in WT Ishikawa cells at any time point investigated. This implies that, as PROKR1 expression is low in these cells, PROK1 induced expression of COX-2 occurs exclusively via activation of PROKR1. Elevation of COX-2 protein expression was also observed from 4 hours, with a peak in protein expression at 8 hours. This suggests that, from 4 hours, PROK1 induced COX-2 protein is available to initiate prostaglandin synthesis.

It is of interest to determine the transcriptional activation of COX-2 by PROK1 as COX-2 mRNA expression has been previously demonstrated to be dependent partially on transcription and partially on stabilisation of the mRNA (Xu K *et al*, 2000, Lasa *et al*, 2000, Mifflin *et al*, 2004). Use of a promoter reporter construct containing the full length COX-2 promoter sequence linked to firefly luciferase revealed maximal transcriptional activation of COX-2 expression by PROK1 at 6 hours. However, an increase in COX-2 transcription of around 2 fold was observed, compared with an increase in COX-2 mRNA expression of approximately 7-fold. Although these two values are not directly related, the observation suggests that PROK1 mediated control of COX-2 expression is not completely transcriptionally regulated. Incubation of PROKR1 Ishikawa cells with 40nM PROK1 in the presence of Actinomycin D, a powerful transcriptional inhibitor, revealed around a 40 - 50% reduction in COX-2 mRNA expression. These data indicate

that PROK1 mediated expression of COX-2 is not completely transcriptionally controlled and stabilisation of COX-2 mRNA may play a role in PROK1 mediated COX-2 expression.

Gene expression is widely recognised to be regulated by up-stream activation of protein kinase cascades (Su & Karin, 1996). Activation of MAPK signalling positively regulates the activities of a number of transcriptional factors (Hill & Treisman, 1995) and post transcriptional control mechanisms (Nair *et al*, 1994). In addition, activation of protein phosphorylation cascades has been demonstrated to activate COX-2 expression (Xu *et al*, 2007, Eckert *et al*, 2007). In Chapter 4 it was demonstrated that PROK1 mediated transient phosphorylation of cSrc, EGFR, ERK 1/2, and activation of ERK 1/2 phosphorylation was dependent on phosphorylation of cSrc and EGFR. In order to investigate if PROK1 induced expression of COX-2 was dependent on activation of protein phosphorylation cascades, chemical inhibitors and dominant negative isoforms of signalling molecules were employed. PROK1 mediated COX-2 promoter activity at 6 hours was inhibited by chemical inhibitors of Gq, PLC-β, Ca^{2+}, cSrc, EGFR or MEK, or co-transfection with dominant negative mutant isoforms of cSrc, EGFR, Ras or MEK. These data suggested that transcriptional activation of COX-2 by PROK1 is dependent on activation of protein phosphorylation cascades leading to phosphorylation of ERK 1/2. However, examination of the effect of chemical inhibitors on PROK1 induced COX-2 mRNA expression at 6 hours revealed that only the Gq inhibitor inhibited PROK1 induced COX-2 mRNA expression to basal levels. Inhibitors of PLC-β, Ca^{2+}, cSrc, EGFR and MEK significantly inhibited COX-2 expression but not to basal levels. These data may imply that activation of the receptor is necessary but PROK1 may induce other signalling phosphorylation cascades (e.g. Other MAPK's such as p38 and JNK) in signalling to COX-2.

Investigation of COX-2 protein expression revealed a significant inhibition of PROK1 induced COX-2 protein expression at 8 hours upon treatment of PROKR1 Ishikawa cells with 40nM PROK1 in the presence of inhibitors of Gq, PLC-β, Ca^{2+}, cSrc, EGFR and MEK. These data indicate that activation of

protein phosphorylation cascades by PROK1 via PROKR1 are important in mediating PROK1 induced COX-2 protein expression.

Stimulation of PROKR1 Ishikawa cells with 40nM PROK1 induced de novo synthesis of PGE_2 and $PGF_{2\alpha}$ with maximal synthesis of both prostaglandins at 12 hours. PROK1 induced synthesis of prostaglandins occurred specifically via COX-2 as incubation of PROK1 treated PROKR1 Ishikawa cells with the specific COX-2 inhibitor NS-398 inhibited the prostaglandin production to basal levels at all time points investigated.

Prostaglandins, the bioactive products of COX-2 metabolism of arachidonic acid, are suggested to be involved in the events of early pregnancy such as decidualisation, vascular permeability and placentation (Cheng and Stewart, 2003, Matsumoto *et al*, 2002). The COX-2 null mouse, in contrast to previous reports (Lim *et al*, 1997), does support blastocyst implantation. However, implantation in the null mice was delayed by approximately 24 hours, with a corresponding reduction in decidual weights and delay in parturition of pups by approximately 24 hours compared with WT mice (Cheng and Stewart, 2003). Although these studies reported differential outcomes in the absence of COX-2 epression, this may be due to different genetic backgrounds. It has been reported that COX-2 null mice on a CD-1 background demonstrate elevated implantation rates compared to mice on a C57BL/6J/129 background due to up-regulation of COX-1 expression. These data may help explain the differences between the two studies.

In the uterus of the COX-2 null mouse, administration of stable analogues of PGI_2 have been demonstrated to rescue the decidualisation defect (Lim *et al*, 1997). However, while PGI_2 is the main prostaglandin in the mouse uterus during early pregnancy, the human endometrium produces little PGI_2 (Abel & Kelly, 1979). PGE_2 is suggested to be of more importance in the human and has been demonstrated to induce decidualisation of human endometrial stromal cells (Dimitriadis *et al*, 2005, Frank *et al*, 1994). The decidualization response induced by PGE_2 is suggested to take place via induction of cAMP production (Kennedy *et al*, 1982, Houserman *et al*, 1989). Therefore the

elevation of COX-2 and PGE_2 production by PROK1 may be important in regulating decidualisation in early pregnancy in humans. Indeed, the prostaglandins which induce the decidualisation response appears to vary considerably among different species (Lim *et al*, 1999, Pakrasi, 1997, Evans & Kennedy, 1978, Wang *et al*, 2004, Kennedy, 1977, Kennedy & Zamecnik 1978).

Initiation of implantation is always associated with an increase in adhesiveness of the epithelium and an increase in vascular permeability. This is associated with an increase in COX-2 expression (Chakraborty *et al*, 1996), along with expression of PGE synthase, PGE_2 and EP receptors at the implantation site (Shi *et al*, 2005, Wang *et al*, 2004, Ni *et al*, 2002). COX-2 null mice however display a reduced vascular permeability at the implantation site compared to WT mice (Matsumoto *et al*, 2002), potentially associated with a deficiency in PGE_2 influenced uterine angiogenesis (Wang and Dey, 2006). Again, via induction of COX-2 and PGE_2 production in the uterus, PROK1 may regulate this increased permeability and direct physiological blood vessel development at the implantation site. Indeed, as has been demonstrated in Chapter 3, PROKR1 is expressed on endothelial cells potentially providing a site for the direct action of PROK1 on the endothelium.

COX-2 is expressed in the human endometrium with localisation to the luminal and glandular epithelium in the secretory phase of the menstrual cycle (Jones *et al*, 1997, Stavreus-Evers *et al*, 2005). COX-2 is also specifically localised to the luminal epithelium and underlying stroma on day 4 of pregnancy in the mouse, spreading to the primary decidual zone by day 5 of pregnancy (Song *et al*, 2000). This expression pattern in the mouse and human supports a role for COX-2 in implantation and decidualisation. It is also suggested that COX-2 expression at the mesometrial pole on day 6 of pregnancy in mice may be important in producing prostaglandins involved in angiogenesis and placenta formation (Chakraborty *et al*, 1996). Indeed, COX-2 can induce expression of VEGF (Joo *et al*, 2003), and this has been further demonstrated in the mouse at the time of implantation (Matsumoto *et al*, 2002).

Another indicator that COX-2 derived prostaglandins are important in implantation and decidualisation is the observation that the prostaglandin inhibitor indomethacin inhibits implantation in rodents (Sookvanichsilp *et al*, 2002, Shafiq *et al*, 2004). Indeed, initial studies performed using non-steroidal anti-inflammatory drugs, which were subsequently identified as inhibitors of prostaglandin synthesis, reported a reduction in edema at the implantation site (Horan *et al*, 1971). Many studies have since elaborated on the role of COX-2 derived prostaglandins in implantation and decidualisation (Biggers *et al*, 1981, Kennedy *et al*, 1990). It has also been reported that prolonged administration of COX inhibitors reduces fertility in humans (Pall *et al*, 2001, Norman & Wu, 2004, Schnitzer *et al*, 2001).

In the human the risk of early pregnancy loss increases as a function of delaying implantation outside the normal window of the receptive endometrium and may result in formation of a defective feto-placental unit (Red Horse *et al*, 2004, Wilcox *et al*, 1999). This may be due to deficient endometrial expression of certain gene products required for implantation, decidualisation and early pregnancy events outside the normal window of receptivity. As COX inhibitors appear to have detrimental effect on human fertility (Pall *et al*, 2001) one of these products may be COX-2 and COX-derived prostaglandins. The parallels with human implantation may be drawn with the delayed implantation observed in the COX-2 null mouse (Cheng and Stewart, 2003) with an associated reduction in decidual weights and a delay in parturition. Expression of COX-2, prostaglandins and appropriate prostaglandin receptors in the human endometrium may therefore be essential for correct 'on-time' implantation (Song *et al*, 2000).

In conclusion, this chapter has highlighted the temporal regulation and mechanism of activation of COX-2 via PROK1-PROKR1 interaction. Expression of COX-2 and downstream prostaglandins, with known roles in implantation and decidualisation, are induced by PROK1 via a PLC-β - Ca^{2+} - cSrc - EGFR - MEK mediated pathway.

Chapter 7 – Prokineticin 1 regulation of LIF expression in PROKR1 Ishikawa cells

7.1. Introduction

In Chapter 5 it was demonstrated that PROK1 elevated expression of leukaemia inhibitory factor (LIF) in PROKR1 Ishikawa cells. LIF expression in the endometrium is suggested to be essential for pregnancy (Stewart *et al*, 1992).

LIF is a pleiotropic cytokine present in many tissues within the human body (Hilton & Gough, 1991, Schafer-Somi, 2003) and is required for diverse functions including neuronal differentiation and maintenance, osteoblast formation and hemopoiesis. LIF is a highly glycosylated protein of 40-50kDa and is a member of the IL-6 family of cytokines along with oncostatin M (OsM), ciliary neurotrophic factor (CNTF), cardiotrophin-1 (CT-1) and IL-11 (Callard, 1994). These cytokines signal through heterodimerisation of their own receptors with the common receptor gp130. LIF binds to its own receptor with relatively low affinity; however heterodimerisation with gp130 forms a high affinity receptor complex and LIF signalling is initiated (Heinrich *et al*, 2002). LIF, also known as interleukin for DA cells (murine IL-3 sensitive cell line), was originally identified via its induction of differentiation in the M1 myeloid leukaemia cell line and its inhibition of differentiation of embryonic cell lines (Hilton *et al*, 1992, Smith *et al*, 1988).

Expression of LIF can be detected in the mouse uterus on day 1 of pregnancy. This expression then disappears, followed by maximal expression in the endometrial glandular cells following the burst of nidatory estrogen on day 4 of pregnancy. However, by day 5 LIF expression has again declined (Bhatt *et al*, 1991). Examination of pseudopregnant mice revealed the same pattern of expression, suggesting that endometrial LIF expression is under maternal control rather than under the influence of the blastocyst (Bhatt *et al*, 1991). In the LIF null mouse model it has been demonstrated that endometrial LIF is essential to implantation (Stewart *et al*, 1992). In contrast to the mouse, LIF expression in the human endometrium is not dependent on a burst of estrogen. The mRNA and protein expression and localization of LIF in the human endometrium across the menstrual cycle have been extensively examined. It has been reported that mRNA

expression of LIF is low in the proliferative phase of the menstrual cycle which then rises to peak in the mid-late secretory phase of the menstrual cycle (Arici *et al*, 1995, Vogiagis *et al*, 1996, Charnock-Jones *et al*, 1994, Kojima *et al*, 1994). LIF protein is detectable only in the mid-late secretory phase of the menstrual cycle and localises to the luminal and glandular epithelial cells (Charnock-Jones *et al*, 1994, Vogiagis *et al*, 1996, Cullinan *et al*, 1996). The mid secretory phase of the menstrual cycle is described as the 'window of implantation' (Psychoyos, 1973). This is the stage of the menstrual cycle when the endometrium becomes receptive, by a number of morphological and molecular mechanisms, to implantation of a blastocyst. Production of LIF from endometrial explants also appears to display elevation in the secretory phase of the menstrual cycle (Laird *et al*, 1997, Delage *et al*, 1995). This production of LIF continues into early pregnancy with LIF measured in explants of the decidualized endometrium of the first trimester of pregnancy (Hambartsoumian, 1998b, Ren *et al*, 1997).

A role for LIF in uterine receptivity has clearly been suggested by the LIF null mouse model and the expression pattern in the human endometrium. A number of factors, including IL-1, TGF-β, EGF and hCG have been shown to induce expression of LIF (Arici *et al*, 1995, Perrier d'Hauterive *et al*, 2004, Licht *et al*, 2001). In chapter 5, LIF was identified as a target gene for PROK1. This study was therefore designed to investigate the temporal regulation and mechanism of LIF expression induced by PROK1 in PROKR1 Ishikawa cells.

7.2. **Materials and methods**

7.2.1. Immunofluorescent histochemistry and confocal microscopy

Co-localization of PROKR1 with LIF was performed by dual immunofluorescence immunohistochemistry on endometrial tissue or first trimester decidua. Sections (5μM) were dewaxed in xylene and rehydrated using decreasing concentrations of ethanol. Antigen retrieval was performed for 5 minutes in a pressure cooker in boiling 0.1% citrate buffer (Ph 6.0). Endogenous peroxidase activity was quenched with 3% (vol/vol) H_2O_2 in methanol at room temperature. Normal horse serum block (5% serum in Tris-buffered saline with 0.05% BSA) was applied for one hour before overnight

incubation at 4°C with goat anti-LIF antibody at 1:50 or goat IgG for control sections. Sections were washed in PBS, LIF treated sections were incubated with biotinylated horse anti goat, followed by washing in PBS and incubation with the fluorochrome Streptavidin 546 (red) diluted 1:200 in PBS. Sections were re-blocked with 5% normal goat serum block diluted in PBS and incubated with rabbit anti-human PROKR1 (1:500) overnight at 4°C. Control sections were incubated with rabbit IgG. Sections were washed in PBS and incubated with peroxidase goat anti-rabbit, 1:200 in PBS, followed by washing in PBS and incubation with the fluorochrome TSA plus Fluorescein (green) at 1:50 in substrate provided with the kit. Sections were washed in PBS and then incubated with the nuclear counterstain ToPro diluted 1:2000 in PBS. Sections were mounted in Permafluor and coverslipped, and fluorescent images were emodellin using a laser-scanning confocal microscope (LSM 510, Carl Zeiss, Jena, Germany) with a 40 x 1.4 aperture oil immersion lens.

7.2.2. Cell culture and treatments

7.2.2.1.Time course analysis of PROK1 mediated LIF expression

In order to examine the temporal regulation of LIF by PROK1, PROKR1 or WT Ishikawa cells were treated with 40nM PROK1 or vehicle for 0, 2, 4, 6, 8, 12 or 24 hours. Cells were subsequently lysed in TRI-reagent, followed by RNA extraction and processing for Taqman quantitative PCR analysis (described in Section 7.2.3)

7.2.2.2. Mechanism of PROK1 mediated LIF expression

In order to examine the signalling pathway activation leading to PROK1 mediated LIF expression, PROKR1 Ishikawa cells were preincubated with chemical inhibitors of Gq protein (YM254890), PLC-β (U73122), Ca^{2+} (calcium chelator, BAPTA-AM), cSrc (PP2), EGFR kinase (AG1478) or MEK (PD98059; concentrations of inhibitors given in Table 15). PROKR1 Ishikawa cells were subsequently stimulated with vehicle or 40nM PROK1 for 6 hours alone or in the presence of the aforementioned signalling inhibitors.

In order to determine if PROK1 mediated LIF expression was transcriptionally activated, PROKR1 Ishikawa cells were preincubated with an inhibitor of transcriptional activation (Actinomycin D, 250ng/ml). PROKR1 Ishikawa cells were treated with 40nM PROK1 for 4, 6 or 8 hours in the presence or absence of Actinomycin D.

Cells were subsequently lysed in TRI-reagent, followed by RNA extraction and processing for Taqman quantitative PCR analysis as described in Section 7.2.3.

Table 15. Inhibitor concentrations

Inhibitor	Molecule Inhibited	Inhibitor concentration
YM254890	Gq protein	1μM
U73122	Phospholipase C	10μM
BAPTA-AM	Calcium	50μM
PP2	c-Src	10μM
AG1478	EGFR	200nM
PD98059	MEK	50μM
Actinomycin D	Transcriptional activation	250ng/ml

7.2.3. Taqman quantitative PCR

7.2.3.1. RNA extraction

RNA was extracted using TRI-reagent and the phenol-chloroform method of RNA extraction as described in section 2.5. Briefly, cells were lysed in TRI-reagent and a homogenous lysate achieved by mixing by pipetting. Lysed samples were then loaded into heavy gel phase lock tubes with the addition of bromo-chloro propane. Tubes were shaken vigorously to ensure adequate mixing of the two solutions. Samples were then processed as described in section 2.5.

7.2.3.2. Taqman quantitative PCR

Expression of RNA within cultured cells was examined by Taqman quantitative PCR analysis as described in section 2.5. Briefly, cDNA was prepared in a random hexamers primed reaction using 200ng per reaction.

Quantitative PCR analysis was performed on the cDNA using specific primer probe combinations to detect LIF (sequence given in Table 5), these were designed in house and custom synthesised. The probe was given a FAM fluorescent label with a TAMRA quencher. Gene expression was normalized by including primer-probe mix, which was Vic labelled, to detect the 18s ribosomal subunit (sequence given in Table 5) as a loading control for the amount of cDNA added in each sample.

Reaction mixes were loaded in duplicate onto a 96-well MicroAmp fast optical reaction plate for analysis on an ABI7900 HT Fast Real-Time PCR machine. Data were analysed and processed using sequence detector version 1.6.3. Results were expressed as relative to a positive RNA standard included in all reactions. The data were analysed using the comparative C_T method for relative quantification.

7.2.4. LIF ELISA

The LIF ELISA has been fully described in section 2.10. In order to examine the temporal regulation of PROK1 mediated LIF protein production, PROKR1 Ishikawa cells were treated with vehicle or 40nM PROK1 for 0, 2, 4, 6, 8, 12 and 24 hours. Medium was removed and frozen until assay. LIF concentration was calculated by constructing a standard curve using LIF standards of known concentration. Cell culture samples were expressed as pg/ml as no LIF secretion was detected in vehicle treated control samples.

7.2.5. Statistics

Statistical analysis of the data in this study was carried out by ANOVA with Fishers PLSD (predicted least squares difference) applied, using Statview 5.0 (Abacus concepts). The data are presented as mean ± SEM of at least 3 experiments.

7.3. Results

7.3.1. Co-expression of PROKR1 and LIF in human endometrium and first trimester decidua

Gene array analysis suggests that expression of endometrial LIF may be under the regulatory control of PROK1 via PROKR1. In order to investigate the potential physiological regulation of LIF expression by PROK1 *in vivo*, tissue localisation of LIF and PROKR1 were examined by fluorescent immunohistochemistry and confocal microscopy. Mid-secretory phase endometrium and first trimester decidua tissue was examined for the expression of PROKR1 and LIF. Expression of LIF (red channel) and PROKR1 (green channel) were localized to the glandular epithelial cells and some stromal cells of the mid secretory endometrium and first trimester decidua (yellow channel, Figure 7.1). LIF expression has been previously demonstrated in the glandular epithelial cells and sub-luminal stroma on day 4 of pregnancy (Fouladi - Nashta *et al*, 2005, Sengupta *et al*, 2003, Song *et al*, 2000). These data suggest that PROK1 - PROKR1 may regulate LIF expression within these cellular compartments in human endometrium and decidua.

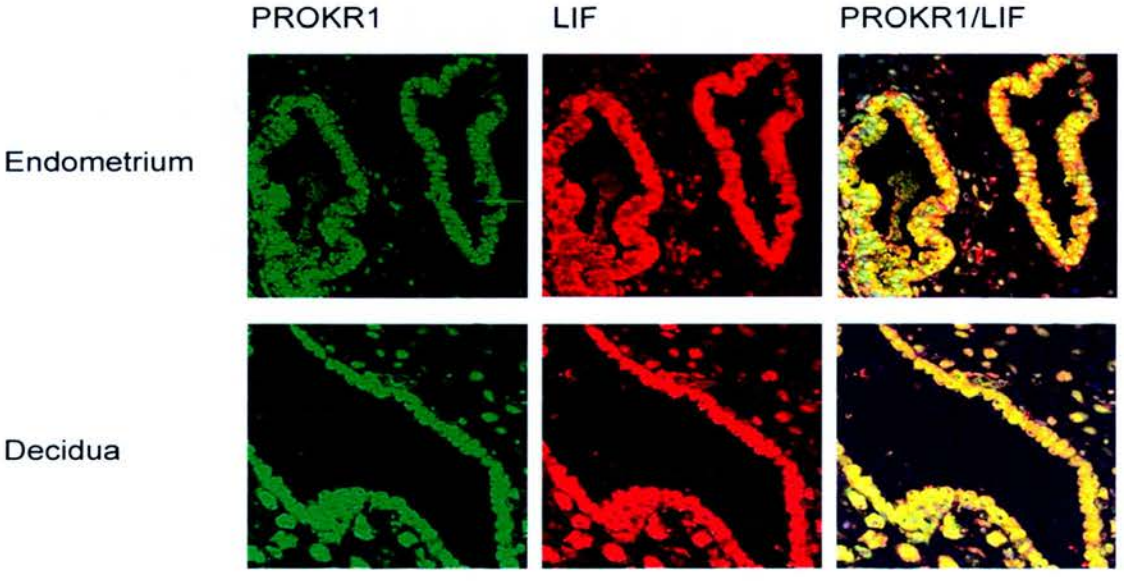


Figure 7.1. Co-localization of PROKR1 and LIF in mid-secretory endometrium and first trimester decidua tissue. Examination of PROKR1 and LIF expression in mid-secretory endometrium and first trimester decidua confirmed co-localisation of PROKR1 (green channel) and LIF (red channel) in glandular epithelial cells of mid-secretory endometrium and first trimester decidua and decidualized stromal cells of first trimester decidua (yellow channel).

7.3.2. Temporal regulation of PROK1 induced LIF expression

7.3.2.1. Temporal regulation of PROK1 induced LIF mRNA expression

Regulation of LIF expression by PROK1 via PROKR1 has been demonstrated by global gene array analysis (section 5.3), and elevation of LIF expression at 8 hours has been independently verified (section 5.3). However, in order to investigate the temporal regulation of LIF RNA expression by PROK1, and therefore elucidate the time of maximal PROK1 induced LIF expression, PROKR1 and WT Ishikawa cells were stimulated with 40nM PROK1 or vehicle for 0, 2, 4, 6, 8, 12 and 24 hours. RNA was extracted and LIF RNA expression examined by Taqman quantitative PCR analysis. PROK1 induced expression of LIF was rapid with an increase in LIF expression of 7.79 ± 1.32 fold by 2 hours, and maximal expression of 17.8 ± 3.8 fold above vehicle treated control by 6 hours ($p<0.001$, Figure 7.2) which declined to almost basal levels by 24 hours. Examination of LIF RNA expression in WT Ishikawa cells treated with 40nM PROK1 for the same time points did not reveal elevation in LIF RNA expression at any time. These data indicate that PROK1 mediated LIF expression is rapid, with maximal LIF expression at 6 hours, which was sustained to 8 hours.

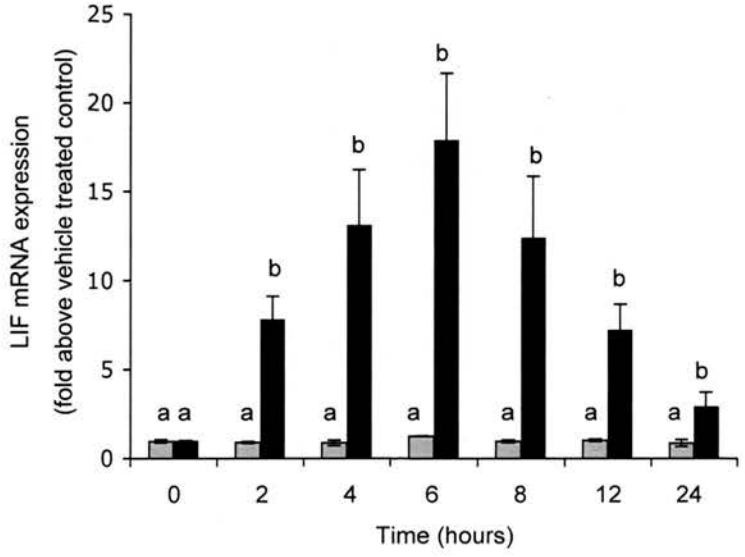


Figure 7.2. Temporal regulation of PROK1 induced LIF RNA expression in PROKR1 and WT Ishikawa cells. PROKR1 and WT Ishikawa cells were treated with vehicle or 40nM PROK1 for 0, 2, 4, 6, 8, 12 or 24 hours. LIF expression was assessed by Taqman quantitative PCR analysis. LIF expression was normalized for loading against expression of 18s and relative to

a single endometrial control. Data are expressed as fold above vehicle treated control. WT Ishikawa cells are represented by grey bars, PROKR1 Ishikawa cells are represented by black bars. LIF expression is rapidly elevated by PROK1 in PROKR1 Ishikawa cells and is maximal at 6 hours. No elevation in LIF expression is evident in WT Ishikawa cells at any time point examined. Data are presented as mean ± SEM. (b is significantly different from a $p<0.001$). **NB**. This figure was presented as part of composite figure 5.2 and has been reproduced here for clarity and interpretation of relevant results within this section.

7.3.2.2. Temporal regulation of PROK1 induced LIF protein production

The temporal regulation of PROK1 mediated LIF secretion was subsequently investigated. PROKR1 Ishikawa cells were treated with 40nM PROK1 or vehicle for 0, 2, 4, 6, 8, 12 and 24 hours. At the designated times, media was removed and LIF protein concentration assessed by ELISA. No LIF protein expression was induced in vehicle treated samples, results are therefore expressed as pg/ml rather than fold above vehicle treated control. LIF protein expression and secretion into culture media was initiated at 4 hours and increased to peak at 12 hours (27.8 ± 3.9 pg/ml, $p<0.05$, Figure 7.3).

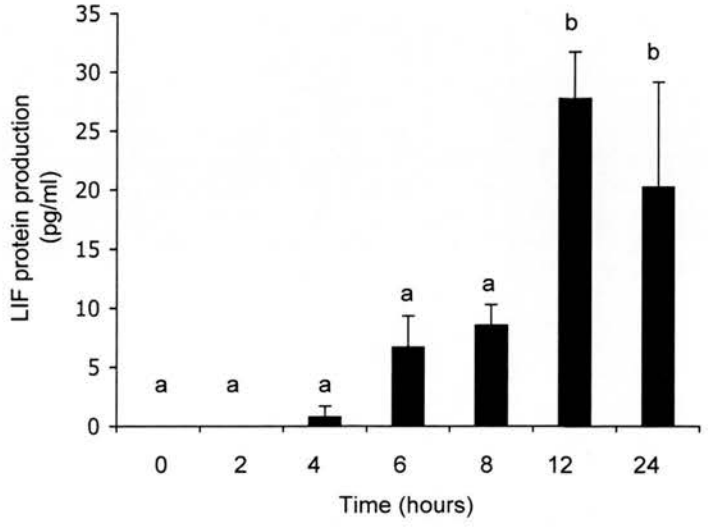


Figure 7.3. Temporal regulation of PROK1 induced LIF protein production in PROKR1 Ishikawa cells. PROKR1 Ishikawa cells were treated with 40nM PROK1 for 0, 2, 4, 6, 8, 12 and 24 hours. LIF protein concentration in cell culture media was assessed by ELISA. LIF production was initiated at 4 hours and production increased to peak at 12 hours followed by a decline in production to 24 hours. Data presented as mean ± SEM. (b is significantly different from a $p<0.05$).

7.3.3. PROK1 induced LIF expression is transcriptionally regulated

In chapter 6 it was demonstrated that PROK1 mediated COX-2 mRNA expression was only partially transcriptionally regulated (section 6.3). However, it has previously been demonstrated that expression of LIF is transcriptionally regulated (Bamberger *et al*, 1997a, Bamberger *et al*, 2004). Therefore, in order to investigate the transcriptional regulation of PROK1 mediated LIF mRNA expression in this system, Actinomycin D, a powerful transcriptional inhibitor was utilised. PROKR1 Ishikawa cells were treated with 40nM PROK1 alone or in the presence of 250ng/ml Actinomycin D for 4, 6, or 8 hours. At 4, 6 and 8 hours, PROK1 mediated LIF mRNA expression was elevated (Figure 7.4). However, this elevation was reduced to basal levels upon co-treatment with Actinomycin D (Figure 7.4). This suggests that in contrast to PROK1 mediated COX-2 expression (Section 6.3), PROK1 induced LIF expression is completely transcriptionally regulated.

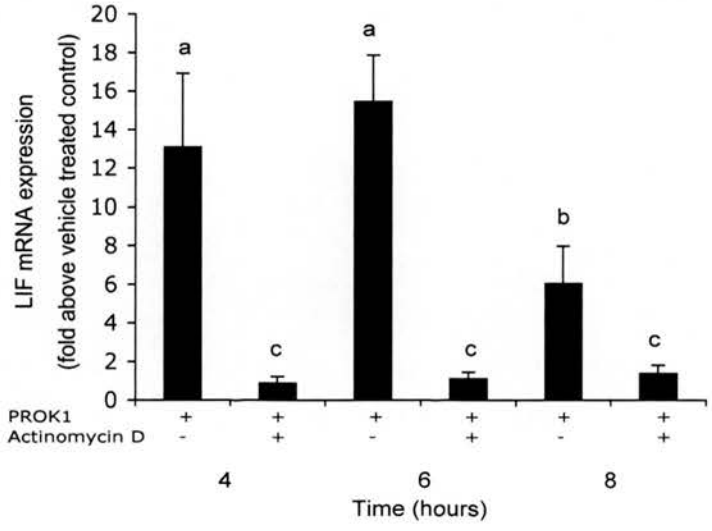


Figure 7.4. PROK1 induced LIF expression is transcriptionally regulated. PROKR1 Ishikawa cells were treated with 40nM PROK1 in the presence or absence of 250ng/ml Actinomycin D for 4, 6 and 8 hours. LIF expression was assessed by Taqman quantitative PCR analysis. LIF expression was normalized for loading against expression of 18s and relative to a single endometrial control. Data are expressed as fold above vehicle treated control. PROK1 induced elevation of LIF RNA expression was completely abolished upon treatment with Actinomycin D, suggesting that PROK1 mediated expression of LIF is transcriptionally regulated. Data are presented as mean ± SEM. + denotes presence of agent, - denotes absence of agent. (c is significantly different from a $p<0.01$, c is significantly different from b $p<0.05$).

7.3.4. Mechanism of PROK1 induced LIF expression in PROKR1 Ishikawa cells

In chapter 4 it was demonstrated that PROK1 induced phosphorylation of ERK 1/2 was dependent on activation of multiple signalling molecules (Section 4.3). It was subsequently demonstrated in Chapter 6 that downstream activation of COX-2 mRNA and protein expression by PROK1 was dependent on activation of these signalling molecules (Section 6.3). In order to investigate the importance of signalling cascade activation in PROK1 mediated LIF expression, PROKR1 Ishikawa cells were treated with 40nM PROK1 in the presence or absence of inhibitors of Gq (YM254890), PLC-β (U73122), Ca^{2+} (BAPTA-AM), cSrc (PP2), EGFR (AG1478) or MEK (PD98059) for 6 hours. LIF mRNA expression was subsequently examined by Taqman quantitative PCR. PROK1 induced elevation of LIF expression at 6 hours (12.44 ± 2.27 fold above vehicle treated control, $p<0.001$, Figure 7.5, lane 2) was significantly inhibited upon co-treatment with PROK1 and inhibitors of Gq protein (Figure 7.5, lane 3), PLC-β (Figure 7.5, lane 4), Ca^{2+} (Figure 7.5, lane 5), cSrc (Figure 7.5, lane 6), EGFR (Figure 7.5, lane 7) and MEK (Figure 7.5, lane 8). These data suggest that PROK1 induced LIF expression is mediated via activation of Gq - PLC-β - Ca^{2+} - cSrc - EGFR and MEK as demonstrated for COX-2 (Section 6.3).

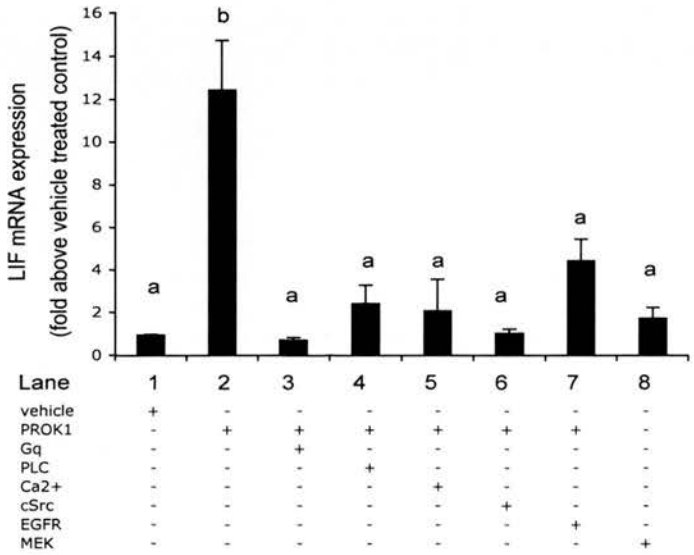


Figure 7.5. Mechanism of PROK1 induced LIF expression. PROKR1 Ishikawa cells were treated with 40nM PROK1 in the presence or absence of inhibitors of Gq protein (YM254890), PLC-β (U73122), Ca^{2+} (BAPTA-AM), cSrc (PP2), EGFR (AG1478) or MEK (PD98059) for 6 hours. LIF expression was assessed by Taqman quantitative PCR analysis

and normalized for loading against expression of 18s and relative to a single endometrial control. Data are expressed as fold above vehicle treated control. Co-treatment of PROKR1 Ishikawa cells with 40nM PROK1 and YM254890, U73122, BAPTA-AM, PP2, AG1478 and PD98059 abolished PROK1 induced elevation of LIF expression at 6 hours. + denotes presence of agent, - denotes absence of agent. Data are presented as mean ± SEM. (b is significantly different from a $p<0.001$).

7.4. Discussion

In Chapter 3, the temporal regulation of PROK1 expression in the endometrium across the menstrual cycle and in early pregnancy was described, with elevation of PROK1 in the secretory phase of the cycle and further elevation in early pregnancy. PROK1 was suggested, by gene array analysis in chapter 5, to regulate LIF expression. Co-localization of LIF and PROKR1 mainly to the glandular epithelium and some stromal cells of mid-secretory endometrium and first trimester decidua (decidualized endometrium of early pregnancy) may imply an autocrine/paracrine signalling mechanism for physiological control of LIF secretion within these cellular compartments *in vivo*. LIF has previously been suggested to localise mainly to the glandular epithelial cells in the mid-secretory endometrium (Charnock-Jones *et al*, 1994). LIF mRNA expression and secretion from endometrial explants also peaks in the mid-secretory phase of the menstrual cycle (Laird *et al*, 1997, Vogiagis *et al*, 1996, Arici *et al*, 1995, Cullinan *et al*, 1996), when PROK1 is also elevated.

LIF production has been reported in first trimester decidua explants (Ren *et al*, 1997) and, as demonstrated in chapter 3 (Section 3.3), PROK1 and PROKR1 are both elevated in first trimester decidua, indicating potential regulation in early pregnancy. Appreciation of the contribution of the endometrial glandular secretions in modulating events at the materno-fetal interface is increasing (Burton *et al*, 2007). PROK1 mediated LIF expression may therefore contribute to these events.

In chapter 5 it was reported that LIF mRNA expression was elevated in PROKR1 Ishikawa cells at 8 hours upon treatment with PROK1. However, whether this represented the peak in PROK1 induced LIF expression was unknown. Stimulation of PROKR1 Ishikawa cells with PROK1 induced a rapid elevation in

LIF mRNA expression with a peak in expression evident at 6 hours, which was sustained to 8 hours. No elevation of LIF mRNA was noted in WT Ishikawa cells upon treatment with PROK1 for the same times. Furthermore, a peak in LIF protein production by PROKR1 Ishikawa cells stimulated with PROK1 was observed at 12 hours, indicating that PROK1 can stimulate production of bioactive LIF which could contribute to endometrial function *in vivo*.

LIF expression induced by IL-1 and TNF is dependent on transcriptional activation as demonstrated by activation of luciferase promoter reporter constructs for LIF (Bamberger *et al*, 1997a, Derigs *et al*, 1993, Carlson *et al*, 1996). In the present study, potential transcriptional activation of LIF by PROK1 was investigated by use of Actinomycin D. Actinomycin D is a powerful inhibitor of transcriptional activity. Co-treatment of PROKR1 Ishikawa cells with PROK1 and Actinomycin D reduced PROK1 induced LIF mRNA expression to basal levels, suggesting that PROK1 induced LIF expression is completely transcriptionally regulated. These data can be contrasted with PROK1 induced expression of COX-2 (Section 6.3), which was only partially transcriptionally activated. These data may indicate that PROK1 regulates expression of different genes via different mechanisms.

As indicated above, activation of LIF expression by IL-1 and TNF is mediated by transcriptional activation (Bamberger *et al*, 1997a, Derigs *et al*, 1993, Carlson *et al*, 1996). However this provides no indication of the signalling cascade that leads to activation of LIF upon ligand-receptor interaction. Elevation of LIF expression induced by IL-1 and TNF has been demonstrated as dependent on activation of Ca^{2+}, PKC and ERK signalling (Carlson *et al*, 1996, Elias *et al*, 1994, Fan *et al*, 2004). LIF activation can also be induced by ionomycin, a potent activator of calcium signalling (Bamberger *et al*, 1997a, 1997b). In chapter 6 it was demonstrated that induction of COX-2 expression by PROK1 was dependent on induction of Ca^{2+} and ERK signalling. Hence the role of this pathway in the regulation of PROK1 mediated LIF expression was investigated. Co-treatment of PROKR1 Ishikawa cells with PROK1 and inhibitors of Gq, PLC-β, Ca^{2+}, cSrc, EGFR and MEK almost completely inhibited PROK1 induced LIF mRNA expression. This suggests that activation of these intracellular signalling molecules

represents the primary signalling cascade mediating PROK1 induced LIF expression.

The LIF null mouse model has provided convincing evidence suggesting a role for LIF in implantation. LIF null males and females are overtly fertile as mating of LIF null males with heterozygous females produces viable blastocysts which implant, develop to term and produce viable adults. Mating of WT or heterozygote males to LIF null females results in production of viable blastocysts, however, the pregnancy does not progress beyond production of the blastocysts and their passage to the uterine lumen as the blastocysts do not implant in the uterine wall (Stewart *et al*, 1992). The blastocysts produced by LIF null females appear to be normal and on days 5-7 of pregnancy they appear to be appropriately located on the antimesometrial side of the uterine lumen (Chen JR *et al*, 2000). However, they develop only loose connections with the luminal epithelium of the uterus and never develop any stronger connections or display any morphological signs indicative of implantation. The luminal epithelium remains intact with no apoptosis evident and no indication of decidualisation. The blastocysts display the characteristics of delayed implantation and furthermore, the loosely connected blastocyst can be retrieved, by uterine flushing, from the uterus up to seven days post coitus with severance of the loose connections it had formed with the uterus (Chen JR *et al*, 2000). There is no apparent problem with these blastocysts as, upon retrieval from the LIF null females and transfer to the uterus of a WT female, even blastocysts 2-3 days past the time of normal implantation could implant and develop normally (Stewart *et al*, 1992). However, blastocyst transfer from a WT female to a pseudopregnant LIF null female does not result in implantation in the LIF null female, strongly suggesting a maternal uterine defect (Stewart *et al*, 1992).

Expression of proteins known to be present in the mouse uterus at the time of implantation have also been examined in the LIF null mouse compared with WT females at the same stage of pregnancy in order to investigate possible interactions and regulatory mechanisms. Compared with the wild-type mice, the LIF null mice do not display a deficiency in ovarian steroids, therefore the absence of implantation cannot be attributed to a defect in the nidatory estrogen surge,

shown to be essential for implantation in mice (Song *et al*, 2000). However, expression of HB-EGF, normally expressed in the luminal epithelium on day 4 and thought to be involved in adhesion and invasion of the trophectoderm, and COX-2, normally present in the luminal epithelium and subluminal stroma and decidual zone on day 4-5 of pregnancy, were aberrantly expressed compared to the expression observed in WT mice (Song *et al*, 2000, Fouladi-Nashta *et al*, 2005). Epiregulin and Amphiregulin, members of the EGF superfamily and HOXA-10, were also aberrantly expressed in LIF null mice (Song *et al*, 2000, Fouladi-Nashta *et al*, 2005). It has been suggested that LIF is at the apex of a signalling cascade controlling expression of these factors. However, data obtained in the present study from gene array analysis of PROK1 treated PROKR1 Ishikawa cells have demonstrated that PROK1 regulates expression of LIF, HB-EGF, Amphiregulin, COX-2 and subsequent downstream prostaglandin production (Chapter 5, Table 12 & Chapter 6). It is therefore tempting to speculate that PROK1 sits at the apex of a signalling cascade regulating implantation events. However, further research is required to outline whether the expression of LIF within the endometrium is solely regulated by PROK1. These data would provide weight to the hypothesis that PROK1 sits above LIF in the hierarchy of signalling molecules involved in implantation. However, given the apparent necessity for LIF expression in implantation, a host of molecules may regulate LIF expression.

The mouse model data may indeed support the role of LIF in implantation. However, LIF is only highly expressed in the mouse uterus after the surge in nidatory estrogen on day 4 of pregnancy (Bhatt *et al*, 1991), whereas in the human endometrium LIF is expressed from the early to the late secretory phase of the cycle with a peak in the mid-secretory phase (Charnock-Jones *et al*, 1994, Vogiagis *et al*, 1996, Arici *et al*, 1995, Cullinan *et al*, 1996). Human endometrial LIF expression is therefore not dependent on an estrogen surge as progesterone is the predominant steroid hormone at the time of implantation.

Does LIF therefore have a proven role in human implantation? There is no definitive answer to this, as clearly a knockout human cannot be produced to test the theory. However, there are a number of lines of evidence, which suggest that LIF is indeed required for human implantation. LIF expression in the human

endometrium peaks in the mid-secretory phase of the cycle (Charnock-Jones *et al*, 1994, Vogiagis *et al*, 1996, Arici *et al*, 1995, Cullinan *et al*, 1996), the putative 'window of implantation', suggested as the time when the endometrium is receptive to implantation by a blastocyst (Psychoyos *et al*, 1973). It has also been observed that women who exhibit endometriosis, unexplained infertility or multiple failures of implantation produce significantly less LIF from endometrial explants or in uterine flushings or display reduced staining for LIF than women who are normally fertile (Laird *et al*, 1997, Delage *et al*, 1995, Hambartsoumian, 1998b, Dimitriadis *et al*, 2006). Mutation in human LIF have also been observed in studies of large cohorts of infertile women (Steck *et al*, 2004, Giess *et al*, 1999, Kralikova *et al*, 2006, Kralikova *et al*, 2006). However, these mutations occur infrequently and appear to be overcome upon application of ovarian stimulation and IVF (Steck *et al*, 2004, Kralikova *et al*, 2007). Additionally, a relationship between the level of LIF expression and pinopod formation has been suggested (Aghajanova *et al*, 2003). These data imply that LIF may have a role in human implantation. Regulation of PROK1 in unexplained infertility has thus far not been investigated however it may be anticipated that if LIF is dysregulated then PROK1 or its receptor may also be aberrantly expressed.

In conclusion, the results from this chapter demonstrate that PROK1 regulates expression of LIF, via transcriptional activation and activation of a Gq - PLC-β - Ca^{2+} - cSrc - EGFR - MEK dependent pathway. PROK1 mediated LIF RNA expression and protein production also appear to occur in a time dependent manner. LIF has a demonstrated role in murine implantation and has been heavily implicated in human reproduction. It is therefore plausible to speculate that PROK1, via induction of LIF expression, may play a significant role in implantation.

Chapter 8 – Physiological relevance of PROK1-PROKR1 signalling in early pregnancy

8.1. Introduction

The data presented thus far is derived from an endometrial epithelial cell line. Detractors may comment that cell lines represent an artificial system. It may also be argued that alteration of the cells by e.g. transfecting receptors into the cells line represents an altered system, which may not prove to be physiologically relevant. In order to demonstrate potential physiological relevance first trimester decidua tissue was used to examine signalling phosphorylation events and gene expression.

First trimester decidua is the endometrial tissue of early pregnancy into which the early embryo invades. The pregnant endometrium supports the growing embryo before it invades the maternal endometrial arteries and gains a blood supply (Burton *et al*, 2007). The decidua is divided into the decidua basalis, which is in contact with the embryo, and the decidua parietalis, which is not in contact. By the method used of collecting first trimester decidua it is unclear whether the tissue is parietalis or basalis, therefore a mixture would be represented.

In chapter 3 it was demonstrated that PROK1 and PROKR1 are elevated in first trimester decidua. PROK1-PROKR1 signalling may therefore be expected to play a role in early pregnancy. In chapter 5, and subsequently in chapters 6 and 7, it was demonstrated that PROK1-PROKR1 induced expression of COX-2 and LIF. These genes are implicated in implantation and potentially in the initial endometrium – blastocyst dialogue. Currently however, there is no experimental evidence indicating initiation of a dialogue between the blastocyst and endometrial PROK1.

Human chorionic gonadotropin is one of the first products of the developing blastocyst. Indeed, hCG subunits are transcribed in the 8-cell embryo and the bioactive hormone is secreted by the blastocyst (Lopata & Hay, 1989) with detection in maternal serum from approximately 14 days post conception (Keay *et al*, 2004). It is increasingly evident that hCG, in addition to its effects on trophoblast differentiation and maintenance of the corpus luteum, has effects

directly on the endometrium (Licht *et al*, 2001). Although hCG detection in maternal serum is a clinical marker of pregnancy, implantation commences approximately 5 - 6 days after conception. Therefore hCG is suggested to have an effect on endometrial physiology well before its detection in maternal circulation.

In the first weeks of pregnancy, hCG is produced in increasing quantities from the syncytiotrophoblast (STB), which is in intimate contact initially with the endometrial luminal epithelium and later with the maternal decidua, as the cells surrounding the invading cytotrophoblast column (Madanes *et al*, 1985, Mishell *et al*, 1973). Through *in vivo* work, infusing hCG into the endometrium of humans and baboons (Licht *et al*, 2001, Sherwin *et al*, 2007), and *in vitro* work, stimulating primary endometrial cells with hCG (Perrier d'Hauterive *et al*, 2004, Han *et al*, 1996, 1999, Zhou *et al*, 1999), it has been demonstrated that hCG induces endometrial expression of a number of genes. These include a selection of those elevated by PROK1 including LIF, COX-2, IL-8, IL-6, and HB-EGF (Licht *et al*, 2001, Sherwin *et al*, 2007, Perrier d'Hauterive *et al*, 2004, Han *et al*, 1996). hCG is suggested to have a role in stimulating endometrial secretion and decidualisation, via induction of COX-2 and downstream prostaglandin production (Han *et al*, 1996, 1999, Zhou *et al*, 1999). HCG has also been suggested to have a reciprocal relationship with endometrial LIF, as LIF elevates trophoblast expression of hCG (Sawai *et al*, 1995a), and hCG, as indicated, elevates endometrial LIF. Both are suggested to induce trophoblast differentiation, as secretion of hCG is a marker of trophoblast differentiation towards syncytialisation. LIF has also been suggested to enhance trophoblast differentiation towards an adhesive or anchoring phenotype rather than an invasive phenotype (Nachtigall *et al*, 1996), with extravillous trophoblasts being invasive. However, conflicting data suggest a role for LIF in trophoblast invasion (Fitzgerald *et al*, 2005, Poehelmann *et al*, 2005).

This study was designed with two aims. First, in order to investigate whether the intracellular signalling cascades and gene expression activated by PROK1-PROKR1 are physiologically relevant, PROK1 mediated ERK 1/2 phosphorylation and gene expression (namely LIF and COX-2) were investigated in first trimester decidua tissue. Secondly, the potential regulation of PROK1 expression and downstream LIF expression in early pregnancy by hCG was investigated.

8.2. Materials and Methods

8.2.1. Tissue Collection

Decidua tissue collection was performed with ethical approval from Lothian Research Ethics Committee under ethics number LREC/05/S1103/32 as indicated in section 2.2. Gestation of pregnancy was confirmed by ultrasound scan prior to elective first trimester surgical termination of pregnancy. Written informed consent was obtained from all subjects prior to tissue collection. Shortly after collection, tissue was placed in RPMI 1640 (containing 2Mm (mmol/litre) L-glutamine, 100U penicillin, and 100μg/ml streptomycin) for tissue culture.

8.2.2. Cell and Tissue Treatments

8.2.2.1.Time course analysis

In order to examine the temporal regulation of PROK1 mediated ERK 1/2 phosphorylation, first trimester decidua tissue explants were treated with 40nM PROK1 or vehicle for 0, 1, 5, 10, 20, 30, 45 or 60 minutes.

In order to determine the temporal regulation of PROK1 induced COX-2 and LIF gene expression and LIF protein production, first trimester decidua explants were treated with 40nM PROK1 for 1, 2, 4, 6, 8, 12 or 24 hours.

In order to determine the temporal regulation of hCG induced PROK1 and LIF gene expression, PROKR1 Ishikawa cells and first trimester decidua tissue were treated with 1IU hCG for 4, 6, 8, 12, 14, 16, 18 or 24 hours.

Cells or tissue were subsequently lysed in NP40 lysis buffer and processed for Western immunoblot analysis (described in Section 8.2.3) or lysed in TRI-reagent, followed by RNA extraction and processing for Taqman quantitative PCR analysis (described in Section 8.2.4).

8.2.2.2.Mechanism of PROK1 induced ERK 1/2 phosphorylation, COX-2 and LIF expression

In order to determine the signalling activated by PROK1 in the signalling cascade to ERK 1/2 phosphorylation, chemical inhibitors of signalling molecules were employed. First trimester decidua tissue explants were preincubated with chemical inhibitors of Gq protein (YM254890), PLC-β (U73122), Ca^{2+} (BAPTA-AM), cSrc (PP2), EGFR kinase (AG1478) or MEK (PD98059; inhibitor concentrations given in Table 16) for one hour prior to stimulation with vehicle or 40nM PROK1 for 30 minutes.

In order to determine the signalling pathway activation leading to PROK1 mediated COX-2 and LIF expression, first trimester decidua tissue explants were pre-incubated with the above inhibitors (inhibitor concentrations given in Table 16) for one hour followed by treatment with vehicle or 40nM PROK1 for 6 hours.

Tissue was subsequently lysed in NP40 lysis buffer and processed for Western immunoblot analysis (described in Section 8.2.3) or lysed in TRI-reagent, followed by RNA extraction and processing for Taqman quantitative PCR analysis (described in Section 8.2.4).

Table 16. Inhibitor concentrations

Inhibitor	Molecule Inhibited	Inhibitor concentration
YM254890	Gq protein	1μM
U73122	Phospholipase C	10μM
BAPTA-AM	Calcium	50μM
PP2	c-Src	10μM
AG1478	EGFR	200nM
PD98059	MEK	50μM
Cyclohexamide	Protein synthesis	10μg/ml

8.2.2.3. Mechanism of hCG induced LIF expression

In order to determine whether production of an intermediary protein is required for hCG mediated LIF expression, PROKR1 Ishikawa cells were pre-incubated with cyclohexamide, an inhibitor of protein synthesis

(concentration indicated in Table 16) for one hour. PROKR1 Ishikawa cells were subsequently treated with 1IU hCG for 8, 12, 16, 18, and 24 hours alone or in the presence of cyclohexamide. Cells were subsequently lysed in TRI-reagent, followed by RNA extraction and processing for Taqman quantitative PCR analysis (described in Section 8.2.4).

8.2.3. Western Immunoblot Analysis

In order to examine ERK 1/2 phosphorylation in first trimester decidua, tissues were finely minced and divided equally for each treatment. Tissue was homogenised in NP40 lysis buffer (described in sections 2.7 and 2.12) and protein concentration assayed as described in section 2.7. 50μg of protein was solubilised in Laemmli protein loading buffer, boiled in order to denature proteins and resolved on an SDS-PAGE gel as described in sections 2.7. Proteins were immunoblotted to a PVDF membrane and non-specific binding blocked with low fluorescence blocking buffer (LiCor). Immunoblotted proteins were visualised by a fluorescent detection system as indicated in section 2.7.

8.2.4. Taqman Quantitative PCR

8.2.4.1.RNA extraction

RNA was extracted using TRI-reagent and the phenol-chloroform method of RNA extraction as described in section 2.5. Briefly, cells or tissue were lysed in TRI reagent and a homogenous lysate achieved by passing the lysate up and down the pipette a number of times. Tissue was lysed by addition of TRI-reagent followed by homogenisation with a tissue lyser (Qiagen). Tissue samples were snap frozen in 2ml Eppendorf tubes, which contained a 5mm steel bead. After addition of TRI-reagent the tubes were loaded onto the tissue lyser and subjected to shaking at 25Hz for 6 minutes, this allowed the tissue to be homogenised by the action of the steel bead. The homogenised tissue samples were centrifuged at 4°C for 10 minutes at 15000 RPM to clear any cellular debris. Lysed samples were then loaded into heavy gel phase lock tubes with the addition of bromo-chloro propane. Tubes were shaken vigorously to ensure adequate mixing

of the two solutions. Samples were then processed as described in section 2.5.

8.2.4.2.Taqman quantitative PCR

RNA expression within cultured cells and first trimester decidua was examined by Taqman quantitative PCR analysis as described in section 2.5. Briefly, cDNA was prepared in a random hexamer primed reaction using 200ng RNA per reaction. Quantitative PCR analysis was performed on the cDNA using specific primer probe combinations to detect COX-2, LIF and PROKR1 (sequence given in Table 5). These were designed in house and were custom synthesised. The probe was given a FAM fluorescent label with TAMRA quencher. Gene expression was normalized by including Vic labelled primer-probe mix to detect the 18s ribosomal subunit (sequence given in Table 5) as a loading control for the amount of cDNA added in each sample.

Reaction mixes were loaded in duplicate onto a 96-well MicroAmp fast optical reaction plate for analysis on an ABI7900 HT Fast Real-Time PCR machine. Data were analysed and processed using sequence detector version 1.6.3. Results were expressed as relative to a positive RNA standard included in all reactions. The data were analysed using the comparative C_T method for relative quantification.

8.2.5. LIF ELISA

The LIF ELISA has been fully described in section 2.8.3. Briefly, first trimester decidua tissue explants were treated with vehicle or 40nM PROK1 for 0, 2, 4, 6, 8, 12 and 24 hours. Medium was removed and frozen until assay. LIF concentration was calculated by constructing a standard curve using LIF standards of known concentration. Sample concentrations of LIF were corrected for the protein content of the sample and expressed as fold above vehicle treated control.

8.2.6. *In vivo* model

This work was performed by JR Sherwin *et al*, and gene array data yielded from this study has been previously published (Sherwin, 2007). Experimental procedures were approved by the Animal Care Committee of the University of Chicago Illinois (Chicago, IL) and performed by A Fazleabas and JM Hastings at the University of Chicago, Illinois. Uterine tissue was obtained from six controls and five hCG-treated adult female baboons (Papio anubis). Ovulation was detected in cycling female baboons by measuring peripheral serum levels of estradiol, beginning 7 days after the first day of menses. The day of the estradiol surge was designated as day -1 with day 0 as the day of the ovulatory LH surge and day 1 as the day of ovulation. On day 5 post ovulation (PO), an oviductal cannula was attached to an Alzet osmotic mini-pump and recombinant hCG was infused at a rate of 1.25 IU/hour for 5 days. The experiment was terminated on day 10 PO (which corresponds to the day of expected implantation in baboon). Total RNA was extracted from each endometrial tissue using TRI reagent as described above. PROK1 mRNA expression was examined by Taqman quantitative PCR analysis as described in Section 8.2.4. JR Sherwin and A Sharkey (University of Cambridge) kindly performed Taqman PCR analysis upon request from J Evans and R Catalano (University of Edinburgh). The results are reproduced here by kind permission of JR Sherwin.

8.2.7. Statistics

Statistical analysis of the data in this study was carried out by ANOVA with Fishers PLSD (predicted least squares difference) applied, using Statview 5.0 (Abacus Concepts). The data is presented as mean ± SEM of at least 3 experiments.

8.3. Results

8.3.1. Intracellular signalling activated by PROK1 in first trimester decidua

8.3.1.1. Time course of PROK1 mediated ERK 1/2 phosphorylation in first trimester decidua

To establish whether PROK1 has a physiologically relevant role, first trimester decidua tissue was utilised to investigate PROK1-PROKR1 signalling. ERK 1/2 phosphorylation was examined in first trimester decidua tissue treated with 40nM PROK1 for 0, 1, 5, 10, 20, 30, 45 and 60 minutes. Maximal ERK 1/2 phosphorylation occurred at 30 - 45 minutes (3.83 ± 0.74 and 3.93 ± 1.21 fold above vehicle treated control respectively, $P<0.01$, Figure 8.1). These data demonstrate that PROK1-PROKR1 signalling is activated in first trimester decidua tissue and may therefore play a role *in vivo*.

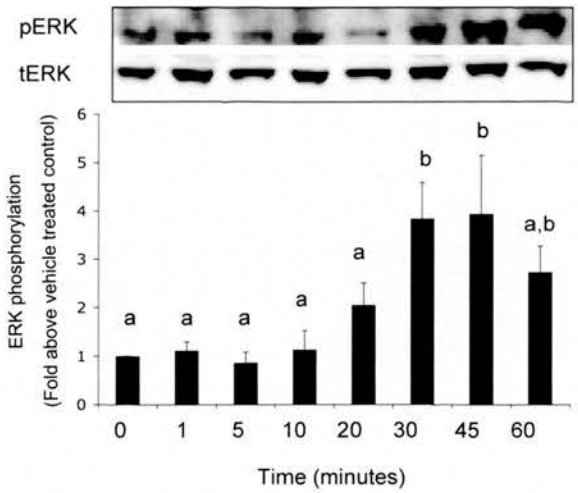


Figure 8.1. PROK1 induced ERK 1/2 phosphorylation in first trimester decidua tissue. First trimester decidua tissue explants were treated with 40nM PROK1 for 0, 1, 5, 10, 20, 30, 45 and 60 minutes. Proteins were extracted and 50μg resolved by SDS-PAGE followed by Western immunoblot analysis using specific antibodies against phosphorylated ERK 1/2 (pERK). The total amount of ERK in cell lysates was determined by probing the same blot with antibody recognising total ERK 1/2 (tERK). ERK 1/2 phosphorylation was normalised against expression of tERK. A representative Western immunoblot is shown with semi-quantitative densitometric analysis. The data are presented as mean ± SEM. (b is significantly different to a, $P<0.01$).

8.3.1.2.Mechanism of PROK1 induced ERK 1/2 phosphorylation in first trimester decidua

In chapter 4 it was demonstrated that PROK1 induced ERK 1/2 phosphorylation was dependent on activation of an intracellular signalling cascade (Section 4.3.3). Therefore, in order to determine whether the signalling phosphorylation cascade activated in PROKR1 Ishikawa cells is physiologically relevant, the PROK1 induced signalling cascade leading to ERK 1/2 activation has been investigated in first trimester decidua explants. Phosphorylation of ERK 1/2 in first trimester decidua is maximal at approximately 30 minutes. PROK1 induced elevation of ERK 1/2 phosphorylation at 30 minutes (5.4 ± 1.19 fold above vehicle treated control, $p<0.001$, Figure 8.2, lane 2) was inhibited by pre-incubation of first trimester decidua explants with inhibitors of Gq protein (Figure 8.2, lane 3) PLC-β (Figure 8.2, lane 4), Ca^{2+} (Figure 8.2, lane 5), cSrc (Figure 8.2, lane 6), EGFR (Figure 8.2, lane 7) and MEK (Figure 8.2, lane 8). These data examining signalling activated by PROK1 in first trimester decidua agree with the data obtained from the PROKR1 Ishikawa cell line. These data suggest that in this primary tissue explant model of early pregnancy, PROKR1 is Gq coupled and activates an intracellular signalling phosphorylation cascade with ERK 1/2 phosphorylation dependent on activation of cSrc and EGFR signalling.

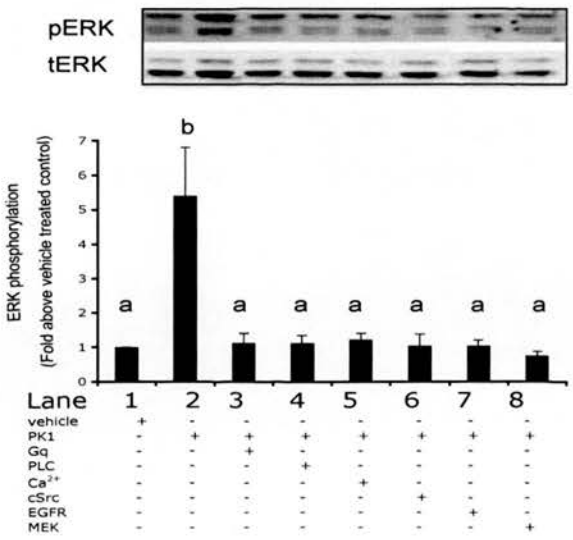


Figure 8.2. Chemical inhibitors of signalling intermediates inhibit PROK1 induced ERK 1/2 phosphorylation. First trimester decidua tissue explants were pre-treated with inhibitors of Gq protein, PLC-β, Ca^{2+}, cSrc, EGFR and MEK for one hour prior to stimulation with 40nM PROK1 for 30 minutes. Proteins were extracted and 50μg resolved by SDS-PAGE followed by Western immunoblot analysis using specific antibodies against phosphorylated ERK 1/2 (pERK). The total amount of ERK in cell lysates was determined by probing the same blot with antibody recognising total ERK 1/2 (tERK). ERK 1/2 phosphorylation was normalised against expression of tERK. + denotes presence of agent, - denotes absence of agent. A representative Western immunoblot is shown with semi-quantitative analysis. The data are presented as mean ± SEM. (b is significantly different to a, $P<0.001$).

8.3.2. Gene expression induced by PROK1 in first trimester decidua

8.3.2.1. PROK1 induces expression of COX-2 mRNA in first trimester decidua

COX-2 expression and prostaglandin production is essential in early pregnancy for control of physiological processes such as decidualisation, vascular remodelling and tone and control of permeability at sites surrounding the blastocyst (Matsumoto *et al*, 2002, Song *et al*, 2000, Lim *et al*, 1997, Cheng & Stewart, 2003). The temporal regulation of PROK1 induced COX-2 expression in first trimester decidua tissue was therefore examined. PROK1 induced COX-2 expression was rapid, with elevation in expression evident from around 2 hours (Figure 8.3). The time point of maximal expression was at 6 hours (2.7 ± 0.5 fold above vehicle treated control, $p<0.05$, Figure 8.3).

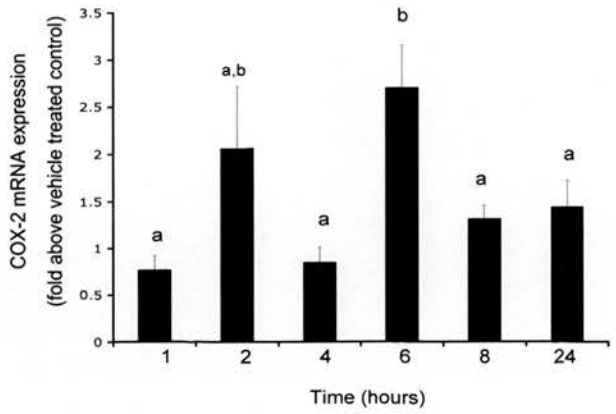


Figure 8.3. Temporal regulation of PROK1 induced COX-2 expression in first trimester decidua tissue. First trimester decidua tissue explants were treated with 40nM PROK1 for 1, 2, 4, 6, 8 and 24 hours. COX-2 expression was assessed by Taqman quantitative PCR analysis. COX-2 expression was normalized for loading against expression of 18s and relative to an internal endometrial control. Data are expressed as fold above vehicle treated control. PROK1 mediated expression of COX-2 was maximal at 6 hours in first trimester decidua tissue. Data are presented as mean ± SEM. (b is significantly different from a $p<0.05$).

8.3.2.2.PROK1 induces expression of LIF mRNA in first trimester decidua

Endometrial LIF expression is essential for implantation (Stewart *et al*, 1992). The temporal regulation of LIF by PROK1 was therefore examined. PROK1 induced LIF RNA expression in first trimester decidua was maximal at 6 hours (2.99 ± 0.53 fold above vehicle treated control, $p<0.05$, Figure 8.4). The PROK1 induced LIF expression then declined almost to basal levels by 8 hours. These data implicate a role for PROK1 in the physiological control of LIF RNA expression in early pregnancy.

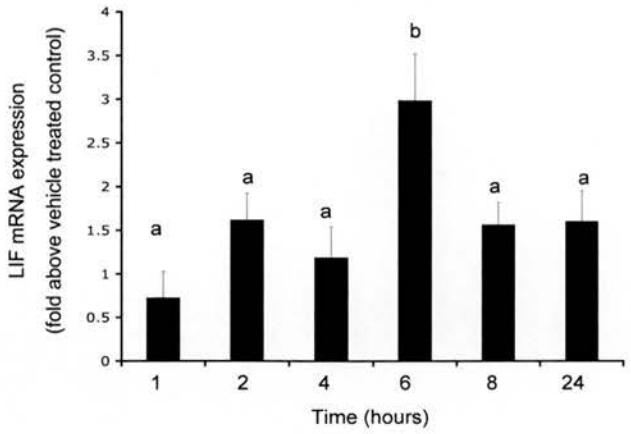


Figure 8.4. Temporal regulation of PROK1 induced LIF RNA expression in first trimester decidua tissue explants. First trimester decidua tissue explants were treated with vehicle or 40nM PROK1 for 0, 2, 4, 6, 8, 12 or 24 hours. LIF expression was assessed by Taqman quantitative PCR analysis. LIF expression was normalized for loading against expression of 18s and relative to an internal endometrial control. Data are expressed as fold above vehicle treated control. LIF expression is rapidly elevated by PROK1 in first trimester decidua and is maximal at 6 hours. Data are presented as mean ± SEM. (b is significantly different from a $p<0.05$).

8.3.2.3.PROK1 induces production of LIF protein in first trimester decidua

Time course investigations in PROKR1 Ishikawa cells have indicated that PROK1 can stimulate the secretion into cell culture medium of LIF protein (chapter 7). In order to determine if this was the case in first trimester decidua, tissue explants were stimulated with vehicle or 40nM PROK1 for 0, 2, 4, 6, 8, 12, 18 and 24 hours. At the indicated times, culture medium was removed and LIF concentration assayed by ELISA. Results are corrected for protein content and expressed as fold above vehicle treated control. LIF production was initiated at 4 hours and production increased to a peak at 12 hours (3.44 ± 1.01 fold above vehicle treated control, $p<0.05$, Figure 8.5) which then declined by 24 hours. This indicates that PROK1 may provide a mechanism of LIF production *in vivo* during early pregnancy.

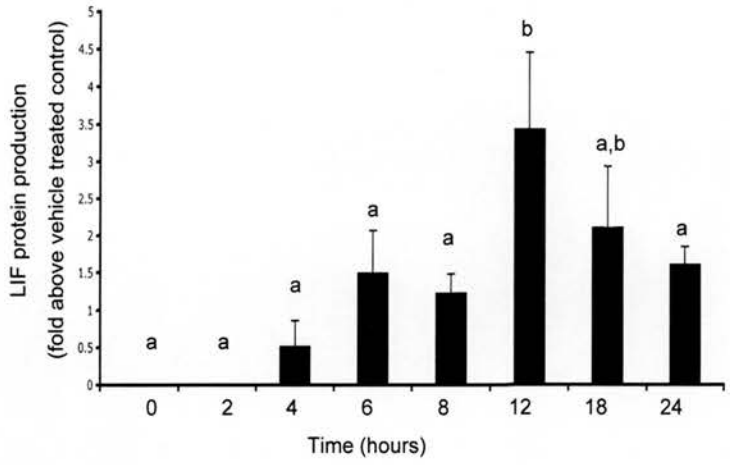


Figure 8.5. Temporal regulation of PROK1 induced LIF protein production in first trimester decidua tissue explants. First trimester decidua tissue explants were treated with 40nM PROK1 for 0, 2, 4, 6, 8, 12, 18 and 24 hours. LIF protein concentration in cell culture medium was assessed by ELISA analysis. The LIF protein secretion was corrected for protein concentration of the sample and expressed as fold above vehicle treated control. LIF production was initiated at 4 hours and production increased to peak at 12 hours followed by a decline in production to 24 hours. Data presented as mean ± SEM. (b is significantly different from a $p<0.05$).

8.3.3. Mechanism of gene expression induced by PROK1 in first trimester decidua

8.3.3.1. Mechanism of PROK1 induced COX-2 expression in first trimester decidua

PROK1 induced COX-2 expression in first trimester decidua is maximal at 6 hours. In order to determine the intracellular signalling pathways activated in PROK1 mediated COX-2 expression, tissue was treated with 40nM PROK1 alone or in the presence of inhibitors of Gq protein, PLC-β, Ca^{2+}, cSrc, EGFR and MEK. PROK1 treatment of first trimester decidua for 6 hours induced a 2.39 ± 0.37 fold increase in COX-2 expression ($p<0.001$, Figure 8.6, lane 2). PROK1 induced COX-2 expression was reduced to basal levels by co-treatment with inhibitors of Gq protein (Figure 8.6, lane 3), PLC-β (Figure 8.6, lane 4), Ca^{2+} (Figure 8.6, lane 5), cSrc (Figure 8.6, lane 6), EGFR (Figure 8.6, lane 7) and MEK (Figure 8.6, lane 8). These data agree with those observed in the PROKR1 Ishikawa cells and suggest that

PROK1 mediated COX-2 expression occurs via a Gq - PLC-β - Ca^{2+} - cSrc - EGFR - MEK mediated pathway.

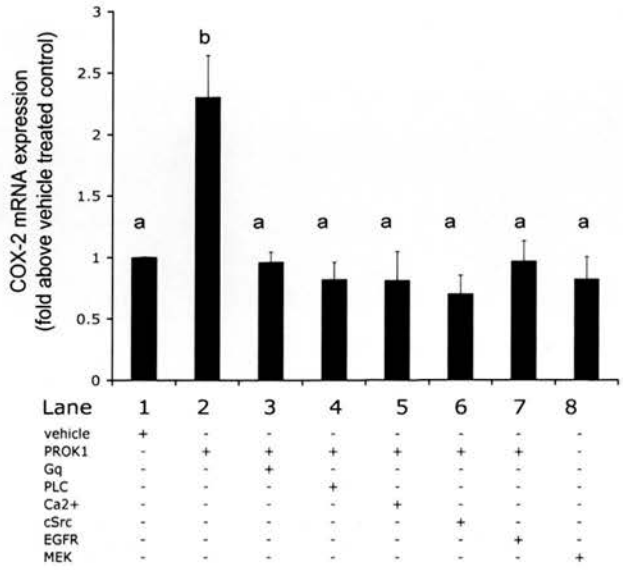


Figure 8.6. PROK1 mediated expression of COX-2 in first trimester decidua is dependent on activation of multiple signalling molecules. PROKR1 Ishikawa cells were treated with 40nM PROK1 in the presence or absence of chemical inhibitors of Gq protein (YM254890), PLC-β (U73122), Ca^{2+} (BAPTA-AM), cSrc (PP2), EGFR (AG1478) or MEK (PD98059) for 6 hours. COX-2 expression was assessed by Taqman quantitative PCR analysis. COX-2 expression was normalized for loading against expression of 18s and relative to an internal endometrial control. Data are expressed as fold above basal levels. Co-treatment with 40nM PROK1 and chemical inhibitors significantly inhibited the maximal PROK1 induced COX-2 expression at 6 hours. + denotes presence of agent, - denotes absence of agent. Data are presented as mean ± SEM. (b is significantly different from a $p<0.001$).

8.3.3.2. Mechanism of PROK1 induced LIF expression in first trimester decidua

As indicated above, in order to correlate the findings obtained using the PROKR1 Ishikawa model cell system with PROK1 signalling *in vivo* first trimester decidua tissue explants were used to determine the mechanism of PROK1 induced LIF expression. First trimester decidua tissue explants were treated with 40nM PROK1 in the presence or absence of inhibitors of Gq protein, PLC-β, Ca^{2+}, cSrc, EGFR or MEK for 6 hours, the time point of maximal PROK1 induced LIF expression in first trimester decidua tissue. Treatment of first trimester decidua with 40nM PROK1 for 6 hours

induced a 2.75 ± 0.56 ($p<0.001$, Figure 8.7, lane 2) fold elevation in LIF mRNA expression (Figure 8.7, lane 2). PROK1 induced LIF expression was abolished upon co-treatment with PROK1 and inhibitors of Gq protein (Figure 8.7, lane 3) PLC-β (Figure 8.7, lane 4), Ca^{2+} (Figure 8.7, lane 5), cSrc (Figure 8.7, lane 6), EGFR (Figure 8.7, lane 7) and MEK (Figure 8.7, lane 8). This indicates that the signalling pathways activated upon PROK1 signalling to LIF demonstrated in the PROKR1 Ishikawa cells may be important *in vivo*.

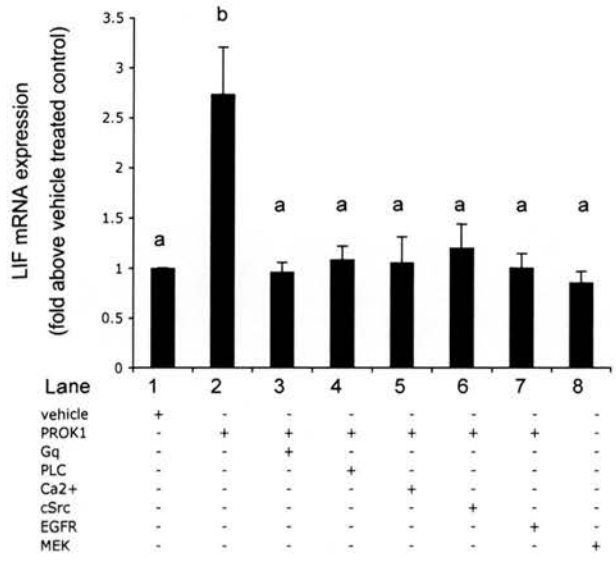


Figure 8.7. PROK1 mediated expression of LIF in first trimester decidua is dependent on activation of multiple signalling molecules. First trimester decidua tissue explants were treated with 40nM PROK1 in the presence or absence of inhibitors of Gq protein (YM254890), PLC-β (U73122), Ca^{2+} (BAPTA-AM), cSrc (PP2), EGFR (AG1478) or MEK (PD98059) for 6 hours. LIF expression was assessed by Taqman quantitative PCR analysis and normalized for loading against expression of 18s and relative to an internal endometrial control. Data are expressed as fold above vehicle treated control. Co-treatment of PROKR1 Ishikawa cells with 40nM PROK1 and YM254890, U73122, BAPTA-AM, PP2, AG1478 and PD98059 abolished PROK1 induced elevation of LIF expression at 6 hours. + denotes presence of agent, - denotes absence of agent. Data presented as mean ± SEM. (b is significantly different from a $p<0.001$).

8.3.4. hCG mediated regulation of PROK1 and LIF expression

8.3.4.1. hCG mediated regulation of PROK1 in PROKR1 Ishikawa cells, first trimester decidua and *in vivo* model

Human chorionic gonadotropin, as one of the earliest embryonic products, may be a candidate for the regulation of PROK1 expression in early pregnancy. Initially the potential temporal regulation of PROK1 expression by hCG was investigated using PROKR1 Ishikawa cells. Treatment of PROKR1 Ishikawa cells with 1IU hCG for 4, 6, 8, 12, 18 and 24 hours induced elevation of PROK1 expression, with maximal expression at 6 hours (3.44 ± 0.66 fold above vehicle treated control, $p<0.01$, Figure 8.8 A). This then declined to basal levels by 18 hours post stimulation with 1IU hCG. In order to determine whether this hCG mediated induction of PROK1 was physiologically relevant two models were used, the first trimester decidua explants already described and a baboon microdialysis model.

First trimester decidua is in intimate contact with the hCG producing syncytiotrophoblast. First trimester decidua would therefore appear to be a good model system in which to examine the potential *in vivo* influence of hCG on endometrial PROK1 expression. First trimester decidua tissue explants were treated with 1IU hCG for 4, 6, 8, 12, 14, 16, 18 and 24 hours. Treatment of first trimester decidua explants with hCG induced a peak in PROK1 expression at 6 hours (1.81 ± 0.2 fold above vehicle treated control, $p<0.05$, Figure 8.8 B). This corresponds with the peak in PROK1 expression induced by hCG in PROKR1 Ishikawa cells.

In the paper of Sherwin *et al* (2007) it was reported that infusion of hCG into baboon endometrium at 1.25 IU/hour for 5 days post ovulation induced elevation in expression of LIF compared to control animals. This was assessed by gene array analysis and validated by real-time PCR. Examination of the same samples using specific primers and probes for baboon PROK1 mRNA revealed a 13.6 fold increase in hCG treated animals compared with control animals (Figure 8.8 C, $P<0.05$). This

demonstrates elevation of PROK1 expression mediated by hCG *in vivo* in the endometrium of a non-human primate

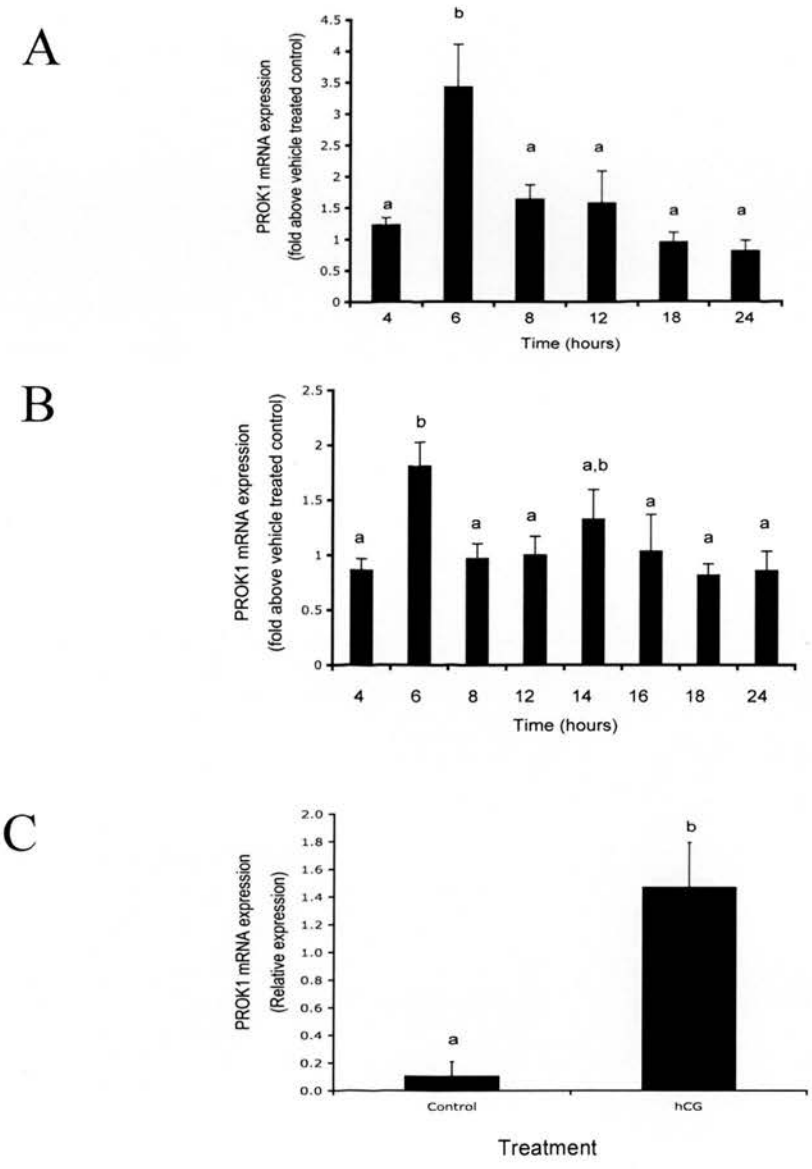


Figure 8.8. Regulation of hCG mediated PROK1 expression. A. PROKR1 Ishikawa cells were stimulated with 1IU hCG for 4, 6, 8, 12, 18 and 24 hours. PROK1 expression was analysed by Taqman quantitative PCR analysis. PROK1 expression is rapidly elevated by hCG in PROKR1 Ishikawa cells and is maximal at 6 hours (b is significantly different from a $p<0.01$). B. First trimester decidua tissue explants were stimulated with 1IU hCG for 4, 6, 8, 12, 14, 16, 18 and 24 hours. PROK1 expression was assessed by Taqman quantitative PCR analysis. hCG induced PROK1 expression is maximal at 6 hours (b is significantly different from a $p<0.05$). C. hCG mediated PROK1 expression *in vivo*. Post-ovulatory baboons were treated with vehicle (control) or 1.25IU hCG per hour for 5 days. PROK1 expression was assessed by Taqman quantitative PCR analysis. PROK1 expression is elevated by hCG in baboon endometrium after 5 days of treatment with hCG (b is significantly different from a

p<0.05). PROK1 expression was normalized for loading against expression of 18s. Data are expressed as fold above vehicle treated control. Data are presented as mean ± SEM.

8.3.4.2.hCG mediated regulation of LIF in PROKR1 Ishikawa cells and first trimester decidua

hCG modulates expression of PROK1 as shown in figure 8.8. In chapters 5, 7 and 8, PROK1 was shown to modulate expression of LIF. hCG has also been demonstrated to regulate expression of LIF (Perrier d'Hauterive *et al*, 2004, Licht *et al*, 2001, Sherwin *et al*, 2007). However, whether hCG regulates expression of LIF via induction of PROK1 is unknown. Here the temporal regulation of LIF expression by hCG is examined. Treatment of PROKR1 Ishikawa cells with 1IU hCG induced an elevation in LIF mRNA expression which was initiated at 12 hours, peaked at 16 hours (2.16 ± 0.2 fold above vehicle treated control, $p<0.05$, Figure 8.9 A) and declined to basal levels by 24 hours. LIF expression is therefore maximal at 16 hours in PROKR1 Ishikawa cells, 10 hours after the peak in PROK1 expression. This temporal difference in expression may suggest that hCG regulates LIF expression via elevation in PROK1 expression.

In order to determine whether this induction of LIF expression by hCG may occur *in vivo*, first trimester decidua explants were again employed. Treatment of first trimester decidua tissue explants with 1IU hCG induced maximal LIF expression at 14 hours (2.35 ±0.4 fold above vehicle treated control, $p<0.05$, Figure 8.9 B), 8 hours after the main peak in PROK1 expression.

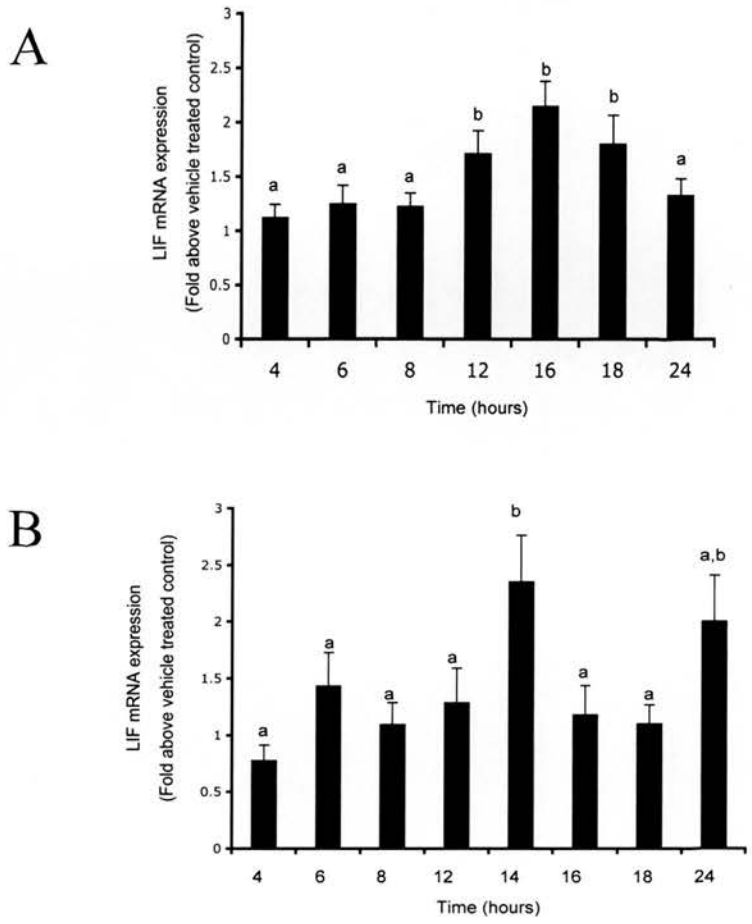


Figure 8.9. Temporal regulation of hCG mediated LIF expression. A. PROKR1 Ishikawa cells were stimulated with 1IU hCG for 4, 6, 8, 12, 16, 18 and 24 hours. LIF expression was assessed by Taqman quantitative PCR analysis. LIF expression is elevated by hCG in PROKR1 Ishikawa cells and is maximal at 16 hours (b is significantly different from a $p<0.05$). B. First trimester decidua tissue explants were stimulated with 1IU hCG for 4, 6, 8, 12, 14, 16, 18 and 24 hours. LIF expression was assessed by Taqman quantitative PCR analysis. LIF expression is elevated by hCG in first trimester decidua tissue explants and is maximal at 14 hours (b is significantly different from a $p<0.05$). LIF expression was normalized for loading against expression of 18s and relative to an internal endometrial control. Data are expressed as fold above vehicle treated control. Data are presented as mean ± SEM.

8.3.4.3.hCG induced LIF expression may be dependent on PROK1

Unfortunately there is no inhibitor of PROK1 commercially available. Therefore, in order to investigate the requirement for an intermediary molecule in hCG induced LIF expression, cyclohexamide was used. Cyclohexamide is a powerful inhibitor of protein synthesis, therefore if a

protein were synthesised by the action of hCG, cyclohexamide would inhibit this synthesis and inhibit any downstream effects of this protein. PROKR1 Ishikawa cells were treated with 1IU hCG alone or in the presence of pre-treatment with 10μg/ml cyclohexamide. hCG induced elevation in LIF expression at 12, 16, 18 and 24 hours was reduced to basal levels in the presence of cyclohexamide. These data indicate that synthesis of an intermediate protein is necessary in order for hCG to stimulate expression of LIF.

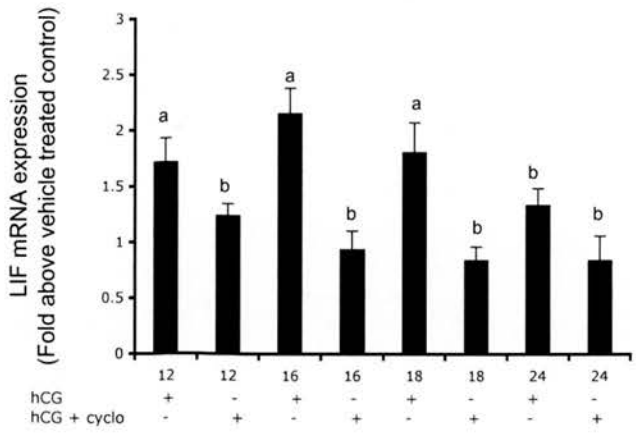


Figure 8.10. hCG mediated LIF expression is sensitive to inhibition by inhibitors of protein synthesis. PROKR1 Ishikawa cells were stimulated with 1IU hCG for 12, 16 18 and 24 hours in the presence or absence of 10μg/ml cyclohexamide. LIF expression was analysed by Taqman quantitative PCR analysis. LIF expression was normalized for loading against expression of 18s and relative to an internal endometrial control. Data are expressed as fold above vehicle treated control. hCG mediated LIF expression in PROKR1 Ishikawa cells and is significantly inhibited in the presence of cyclohexamide. + denotes presence of agent, - denotes absence of agent. Data presented as mean ± SEM. (b is significantly different from a $p<0.01$).

8.4. Discussion

The data presented in Chapters 4, 5, 6 and 7 have demonstrated that PROK1-PROKR1 induces phosphorylation of ERK 1/2 and expression of COX-2 and LIF. However, all these studies were conducted in Ishikawa cells, an endometrial epithelial cell line, transfected with PROKR1. As this may represent an altered, non-physiological system, the signalling pathways and gene expression mediated by PROK1 were investigated in first trimester decidua. First trimester decidua is

the decidualized endometrium of early pregnancy into which the embryonic tissues invade and remodel in order to support the developing embryo and connect with the maternal blood supply.

Treatment of first trimester decidua tissue explants with PROK1 induced significant ERK 1/2 phosphorylation at 30 - 45 minutes which was subsequently shown to be dependent on Gq - PLC-β - Ca^{2+} - cSrc, EGFR and MEK. These data correspond with that obtained from the PROKR1 Ishikawa cells (Section 4.3) suggesting the data obtained from these cells may indeed represent physiological endometrial responses. However, the peak in PROK1 induced ERK 1/2 phosphorylation in first trimester decidua was considerably delayed compared with the peak in PROKR1 Ishikawa cells (30 minutes in decidua vs. 5 minutes in cells). This may represent differences in comparing a homogenous cell monolayer with a tissue explant consisting of a heterogeneous cell population, some of which will express receptors and some not, possibly with differential levels expressed in different cell types.

In order to demonstrate that the gene responses elicited by PROK1 are potentially relevant *in vivo*, COX-2 and LIF expression were also examined in first trimester decidua. Again the results in the first trimester decidua correlated with those obtained from the PROKR1 Ishikawa cell line with a peak in COX-2 and LIF mRNA expression at 6 hours and a peak in LIF protein production at 12 hours. In the PROKR1 Ishikawa cell line, the inhibitors of Gq, PLC-β, Ca^{2+}, cSrc, EGFR and MEK significantly inhibited PROK1 mediated mRNA expression of COX-2 and LIF. Studies in the first trimester decidua explants agree with these data with the expression of COX-2 and LIF inhibited to basal levels by co-treatment with PROK1 and chemical inhibitors of signalling molecules. These data again suggest that the data obtained from the cell line is physiologically relevant and activation of Gq - PLC-β - Ca^{2+} - cSrc - EGFR and MEK are essential in PROK1 mediated COX-2 and LIF expression during early pregnancy.

It must be considered that, as the first trimester decidua tissue explants are not a homogenous cell population expressing PROKR1, a proportion of the response induced by PROK1 may be transmitted via PROKR2. PROKR2 is also expressed

in the endometrium (Battersby *et al*, 2004a) and PROK1 can activate PROKR2 (Lin DC *et al*, 2002). Indeed, the two Prokineticin receptors display 85% amino acid identity and are effectively identical in their transmembrane domains, and both ligands can initiate signalling at both receptors (Lin DC *et al*, 2002, Masuda *et al*, 2002, Soga *et al*, 2002). In order to address the contribution of the two receptors specific receptor antagonists would be required.

This study was designed to address two issues, the physiological relevance of the phosphorylation cascades and gene activation mediated by PROK1, and the potential regulation of PROK1 expression in early pregnancy. The physiological relevance of the data obtained from the PROKR1 Ishikawa cells has been demonstrated in first trimester decidua. These data further suggested a role for PROK1 and prokineticin receptors in early pregnancy. However, the regulatory mechanisms governing PROK1 expression in early pregnancy are unknown. PROK1 is elevated in first trimester decidua, as demonstrated in Chapter 3. Progesterone regulates expression of PROK1 in the endometrium (Battersby *et al*, 2004a) and the progesterone secreting corpus luteum is maintained in early pregnancy. It is not clear, however, whether the elevation in PROK1 expression in first trimester decidua is due simply to maintained progesterone secretion. A likely candidate for regulation of PROK1 expression during early pregnancy may be chorionic gonadotropin.

Human chorionic gonadotropin (hCG) is one of the earliest embryonic products, with bioactive hormone secreted from the blastocyst stage (Lopata & Hay, 1989). The 'traditional' role of hCG is considered to be its role in maintaining progesterone production from the corpus luteum in early maternal recognition of pregnancy. However, direct paracrine effects on the endometrium aiding implantation are now suggested to precede these endocrine events (Licht *et al*, 2001, Sherwin *et al*, 2007). A reciprocal relationship between certain endometrial cytokines and hCG has been suggested both *in vitro* and *in vivo*. *In vitro* research, using primary first trimester trophoblast cells, has demonstrated that LIF induces secretion of hCG (Sawai *et al*, 1995a, Ren *et al*, 1997) and functional differentiation of the trophoblast into syncytiotrophoblast (Sawai *et al*, 1995b). *In vivo* research, in which hCG was infused into the endometrium of baboon or humans, and *in*

vitro research, in which primary endometrial cells were treated with hCG, have shown that hCG induces expression of LIF (Licht *et al*, 2001, Sherwin *et al*, 2007, Perrier d'Hauterive *et al*, 2004).

As indicated in chapters 5 and 7, PROK1 regulates expression of LIF mRNA and protein. PROK1 is elevated in first trimester decidua, a tissue in contact with, and under the influence of the hCG secreting syncytiotrophoblast. It was therefore of interest to investigate the potential regulation of PROK1 expression by hCG in order to determine if this regulates PROK1 expression in early pregnancy and represents a step in hCG induced LIF expression. This may indicate a potential role of PROK1 in the reciprocal communication between the embryo and endometrium. As demonstrated in the three different model systems, the PROKR1 Ishikawa cell, first trimester decidua explants and *in vivo* baboon hCG infusion model, hCG induces expression of PROK1 mRNA. Using the PROKR1 Ishikawa cells and first trimester decidua explants, the temporal regulation of hCG induced PROK1 expression was examined. hCG induced PROK1 expression was maximal in PROKR1 Ishikawa cells and first trimester decidua at 6 hours. The hCG infusion into the baboon endometrium was performed for 5 days with infusion of 1.25 IU of hCG per hour. Treatment of baboon endometrium with hCG for 5 days induced a 13.6 fold elevation in PROK1 mRNA expression. Elevation of LIF has previously been demonstrated in the baboon model (Sherwin *et al*, 2007). Although a peak in hCG induced PROK1 expression was observed after just 6 hours in the PROKR1 Ishikawa cells and first trimester decidua, sustained elevation of PROK1 may require sustained delivery of hCG, therefore the baboon hCG infusion model is probably the most physiologically relevant system as, *in vivo*, the syncytiotrophoblast would be constantly releasing hCG. However, the *in vitro* models do tell us that hCG induced expression of PROK1 is rapid.

LIF expression in response to hCG is observed 14-16 hours after treatment with hCG in PROKR1 Ishikawa cells and first trimester decidua. It has been demonstrated in PROKR1 Ishikawa cells and first trimester decidua explants, that PROK1 mediated LIF expression peaks at 6 hours. Therefore a delay in LIF expression of 10 hours after the peak in PROK1 expression should provide enough

time for production and secretion of PROK1 protein in order to stimulate LIF expression.

It is tempting to speculate, in light of the temporal regulation of PROK1 and LIF by hCG, that it is necessary for hCG to induce expression of PROK1 prior to induction of LIF expression. In order to prove this hypothesis an antagonist for PROKR1 or an siRNA directed against PROK1 is required. During this study, these tools were not available. An inhibitor of protein synthesis, cyclohexamide, was therefore used. Cyclohexmide would inhibit protein production induced by hCG and therefore inhibit downstream signalling mediated by any proteins which would have been produced. Co-treatment of PROKR1 Ishikawa cells with hCG and cyclohexamide reduced hCG mediated LIF expression to basal levels, suggesting protein production, potentially production of PROK1, is required for hCG induced expression of LIF.

What relevance does this have for the initiation of pregnancy? LIF has been demonstrated, by the use of the LIF null mouse model, to be essential for implantation (Stewart *et al*, 1992). However, LIF is produced by the human endometrium as a function of secretory transformation (Cullinan *et al*, 1996, Arici *et al*, 1995, Charnock-Jones *et al*, 1994, Vogiagis *et al*, 1996) and is available to facilitate implantation without the aid of hCG. It has therefore been suggested that, by elevating LIF expression, hCG may prolong the period during which the endometrium is receptive (Goto *et al*, 2007, Filicori *et al*, 2005, de Ziegler *et al*, 2004), extending the implantation window and optimising the chances of implantation.

HCG has been demonstrated to modulate other factors associated with early pregnancy, including IL-6, HB-EGF and COX-2 in the baboon endometrium (Sherwin *et al*, 2007). HCG elevates expression of COX-2 and downstream prostaglandin production in endometrial cells during decidual transformation (Han *et al*, 1996, 1999, Zhou *et al*, 1999), thereby contributing to decidualization. HB-EGF is thought to mediate attachment of the blastocyst to the endometrium (Raab *et al*, 1996, Leach *et al*, 1999, Yoo *et al*, 1997, Chobotova *et al*, 2002a, 2002b), while IL-6 is suggested to be involved in implantation and placental

growth (Nishino *et al*, 1990, Robertson *et al*, 2000). As these are also PROK1 regulated genes, their induction by hCG may be dependent on intermediate PROK1. As the contribution of the endometrial glandular products in supporting the developing trophoblast is increasingly understood (Burton *et al*, 2007), it has been demonstrated that trophoblast hCG promotes the secretory transformation of the endometrium (Fazleabas *et al*, 1999), providing a support system for its own growth. Although not demonstrated directly here, PROK1 may be one of those secretory products induced by hCG in the epithelial glandular cells. In Chapter 3 it was demonstrated that PROK1 localises to the glandular epithelial cells of the secretory endometrium and first trimester decidua, the cellular compartment to which the LH/CG receptors have also been localised (Bukovsky *et al*, 2003).

However, hCG production, and therefore its influence on the endometrium, decreases by the second trimester of pregnancy (Hay *et al*, 1988, Gonen *et al*, 1992, Wenstrom *et al*, 1994), suggesting that these roles are specific to the initial implantation and maintenance of pregnancy. These data implicate PROK1 mediated signalling and gene expression in early pregnancy. A role for COX-2, LIF, IL-11 and IL-6 in pregnancy and decidualization is suggested by the reproductive phenotypes of their null mouse models (Lim *et al*, 1997, Cheng & Stewart, 2003, Stewart *et al*, 1992, Robertson *et al*, 2000, Robb *et al*, 1998). These data may implicate a role for PROK1 in implantation and early placental development.

PROK1 may represent the tip of a signalling and gene expression cascade essential for regulating pregnancy related functions such as implantation and possibly decidualization and placentation. However the 'master controllers' regulating PROK1 expression, and therefore genes downstream of PROK1, appear to be the hormones progesterone and hCG. PROK1 expression is regulated by progesterone as previously demonstrated by Battersby *et al* (2004a). Herein it is demonstrated that hCG, produced in significant quantities in early pregnancy (Madanes *et al*, 1985, Mischell *et al*, 1973), regulates expression of PROK1 in the endometrium. These data provide an insight into the complexity of the mechanisms controlling endometrial receptivity, initiation and propagation of pregnancy. Progesterone may control expression of PROK1 during the secretory phase of the menstrual

cycle, thereby mediating endometrial receptivity via induction of genes associated with receptivity by PROK1. In the event of pregnancy, hCG may contribute to the elevation of endometrial PROK1 and therefore downstream gene activation maintaining the opening of the window of implantation. As implantation requires both a receptive endometrium and an appropriately developed blastocyst (Paria *et al*, 1993), PROK1, by influencing factors associated with endometrial receptivity and being under the influence of hCG, may participate in the reciprocal relationship between the endometrium and the blastocyst contributing to reproductive success.

Chapter 9 – Final discussion, conclusions and future work

9.1. Final discussion

9.1.1. Expression and localisation of PROK1 in the non-pregnant and pregnant endometrium

A role for endometrial PROK1 in implantation has been proposed due to its temporal regulation across the menstrual cycle. Within the endometrium, previous studies have localised expression of PROK1 by immunohistochemistry to the epithelial, stromal and endothelial cells, with maximal expression during the secretory phase (Battersby *et al*, 2004a, Ngan *et al*, 2006).

Initial studies for this thesis were performed to examine the expression of PROK1 and its GPCR, PROKR1 across the menstrual cycle, in the pregnant endometrium and in the pathological endometrium of endometrial cancer. These studies demonstrated that PROK1 was elevated during the secretory phase of the menstrual cycle, in agreement with previous studies (Battersby *et al*, 2004a, Ngan *et al*, 2006). It was also found that PROK1 and PROKR1 expression were elevated in the pregnant endometrium compared with the non-pregnant endometrium, while expression of PROK1 is down-regulated in endometrial cancer compared with the secretory phase of the menstrual cycle.

Immunohistochemical analysis demonstrated, in agreement with other studies, expression of PROK1 in the glandular epithelium and stromal compartment with some expression in the endothelial cells of the microvasculature of the non-pregnant endometrium. This study also demonstrated localisation of PROK1 to these compartments in the pregnant endometrium. Similarly, immunohistochemical localisation of PROKR1 demonstrated expression of PROKR1 in the glandular epithelium, stroma and endothelial cells of the non-pregnant and pregnant endometrium. Examination of PROK1 and PROKR1 localisation in endometrial cancer, however, demonstrated minimal expression of either protein in well, moderately or poorly differentiated carcinoma samples.

Expression of PROKR1 is localised to the endothelial cells of the endometrium, potentially suggesting a direct effect of PROK1 on endothelial cells. Expression of PROK1 and PROKR1 is demonstrated in macrophages with expression of PROK1 also localised to the uterine natural killer (uNK) cells. These cells cluster around the invading trophoblast and the maternal blood vessels (Trundley & Moffett, 2004). It is proposed that signals released by uNK cells aid in the remodelling of the maternal blood vessels (Guimond *et al*, 1997). Therefore, PROK1 may contribute to the endometrial endothelial remodelling during pregnancy. This has been demonstrated for other factors, such as VEGF, released by the uNK cells (Hanna *et al*, 2006). However, if PROK1 does contribute to endometrial endothelial remodelling it appears to occur only during physiological processes, not pathological, as PROK1 expression in downregulated in endometrial cancer compared with secretory phase endometrium. Prokineticins are proposed to regulate macrophage activation (Martucci *et al*, 2005). Macrophages are thought to play a role in the tolerance of the invading trophoblast (Rai & Regan, 2002). Therefore, through potentially regulating the activation status of the macrophages, and thereby the cytokines that they secrete (Gordon *et al*, 1995), PROK1 may contribute to maternal tolerance of the invading foetus.

Immunohistochemical analysis also demonstrated expression of PROK1 and PROKR1 in the early placental tissues. In agreement with previous studies, expression of PROK1 localised to the syncytio- and cytotrophoblast layers of the early invading placenta (Hoffmann *et al*, 2006). Expression of PROKR1 also localised to these trophoblast layers, again indicating potential for autocrine or paracrine signalling during early pregnancy and a possible role for PROK1-PROKR1 in maternal-fetal dialogue. Expression of PROK1 by the trophoblast may initiate a communication with the endometrium and guide the trophoblast or maternal blood vessels in order to develop the fetal blood supply. This type of trophic system has been suggested for other factors such as VEGF (Carmeliet, 2005).

These initial data indicated a potential autocrine or paracrine signalling mechanism for PROK1 *in vivo*, during the secretory phase of the cycle and

during early pregnancy. PROK1 has previously been implicated in the chemotaxis of endothelial cells (LeCouter *et al*, 2001), and may therefore play a role in blood vessel maturation and repair during the secretory phase of the cycle or during early pregnancy, when the blood vessels are actively invaded by the fetal trophoblast.

9.1.2. PROK1 mediated intracellular signalling

In order to investigate further the role of PROK1-PROKR1 in the endometrium, an endometrial epithelial cell line (Ishikawa) stably expressing PROKR1 was produced. Elevated expression of PROKR1 in these cells compared with PROKR1 expression in wild type cells was confirmed by PCR analysis. Previous studies have shown that upon activation of prokineticin receptors, inositol phosphate and calcium are mobilised leading to ERK 1/2 phosphorylation (Lin DC *et al*, 2002, Lin R *et al*, 2002, Soga *et al*, 2002, Masuda *et al*, 2002). Therefore inositol phosphate mobilisation and ERK 1/2 phosphorylation were initially examined in order to demonstrate functionality of this receptor within the transfected cells. PROK1 significantly elevated inositol phosphate mobilisation and ERK 1/2 phosphorylation upon treatment with 40nM PROK1 suggesting the receptors were functional.

PROKR1 is a GPCR (Li M *et al*, 2001, Lin DC *et al*, 2002, Lin R *et al*, 2002, Soga *et al*, 2002, Masuda *et al*, 2002). In this study, PROK1-PROKR1 interaction induced inositol phosphate mobilisation and ERK 1/2 phosphorylation, but not cAMP production, suggesting Gq coupling. The ERK 1/2 phosphorylation induced by PROK1 was not inhibited by pertussis toxin, suggesting that PROKR1 is not Gi coupled in these cells. Activation of GPCR's induces down-stream activation of phosphorylation cascades (Werry *et al*, 2005). Investigation of the pathway regulating PROK1 mediated ERK 1/2 phosphorylation revealed that activation of Gq protein, PLC-β, Ca^{2+}, cSrc, EGFR and MEK was required in PROKR1 Ishikawa cells and first trimester decidua. It was subsequently demonstrated that PROK1 induced phosphorylation of cSrc and EGFR, with phosphorylation of cSrc apparently preceding EGFR phosphorylation. These data suggest that the signalling

pathways activated by PROK1 in PROKR1 Ishikawa cells are physiologically relevant and may represent the *in vivo* situation during early pregnancy.

9.1.3. PROK1 mediated gene expression

There have been no previous reports demonstrating gene expression induced upon PROK1-PROKR1 interaction in the endometrium or any other tissue. Therefore in an attempt to determine PROK1 target genes in the endometrium, global gene array analysis was performed on two platforms, using RNA extracted from PROKR1 Ishikawa cells treated with vehicle or 40nM PROK1 for 8 hours.

277 genes were found to be changed by 1.5 fold or more on both platforms, of which 225 were found to be up-regulated with 52 down-regulated. 49 genes were found to be significantly altered by $p \leq 0.05$. A large number of genes regulated by PROK1 have potential roles in endometrial receptivity and implantation, as demonstrated by the reproductive phenotype of mice with null mutations for these genes or investigation of their expression in human or animal endometrium.

Five genes were selected from the list of 49 significantly changed genes for validation. These were COX-2, LIF, IL-6, IL-8 and IL-11. In the validation experiments at 8 hours, these genes were elevated in PROKR1 Ishikawa cells. Examination of the temporal regulation of COX-2, LIF, IL-6, IL-8 and IL-11 demonstrated maximal elevation of all genes between 6 - 12 hours in PROKR1 Ishikawa cells. Importantly, no elevation in gene expression was observed in WT Ishikawa cells in which, as demonstrated in Chapter 4, PROKR1 expression is minimal. These data suggested that genes regulated by PROK1 on the gene array occur via activation of PROKR1.

Follow up studies focused on the examination of PROK1 mediated expression of COX-2 and LIF. Expression of both COX-2 and LIF co-localised with PROKR1 mainly to the glandular epithelium of mid-secretory and pregnant endometrium, potentially suggesting regulation within this compartment. The temporal expression of COX-2 and LIF induced by PROK1 revealed both

factors to be expressed maximally at 6 hours in PROKR1 Ishikawa cells and in first trimester decidua explants, suggesting the cell line data to be representative of the *in vivo* response. Further investigation of the regulation of LIF and COX-2 by PROK1 revealed LIF to be transcriptionally regulated, while COX-2 appeared to be only partially transcriptionally regulated. These data may imply that PROK1 mediated COX-2 expression may derive in part from RNA stabilisation mechanisms, as has been reported in other systems (Xu *et al*, 2000, Lasa *et al*, 2000, Mifflin *et al*, 2004).

Investigation of the signalling pathways that lead to PROK1 mediated COX-2 and LIF expression in PROKR1 Ishikawa cells revealed that, similar to PROK1 induced ERK, gene expression was dependent on activation of Gq - PLC-β - Ca^{2+} - cSrc - EGFR - MEK, as summarised in Figure 9.1. Examination of the signalling pathway leading to PROK1 mediated LIF and COX-2 RNA expression in first trimester decidua revealed inhibition of PROK1 induced LIF and COX-2 to almost basal levels upon treatment with inhibitors of the above signalling molecules, suggesting these data to be of importance *in vivo*. Activation of LIF expression has been demonstrated as dependent on activation of calcium and ERK 1/2 signalling (Bamberger *et al*, 2004, 1997b, Carlson *et al*, 1996, Elias *et al*, 1994, Fan *et al*, 2004). Therefore, the pathway leading to LIF activation presented herein appears to correspond with that observed previously. Similarly, COX-2 expression has previously been demonstrated as dependent on activation of PLC (Luo *et al*, 2003) and RTK's (Xu *et al*, 2007, Liu *et al*, 2007), therefore, the data presented herein again agree with previous studies.

PROK1 induced COX-2 protein expression in PROKR1 Ishikawa cells was temporally regulated, with a peak in expression at 8 hours. This was followed by a peak in prostaglandin production in PROKR1 Ishikawa cells at 12 hours, which was dependent on activation of COX-2. These data suggest that PROK1, via induction of COX-2 expression can induce production of prostaglandins *in vivo*. Prostaglandins are suggested to have multiple roles in female reproduction. PROK1 mediated LIF protein production peaks in both

PROKR1 Ishikawa cells and first trimester decidua at 12 hours, demonstrating that PROK1 can induce production of bioactive LIF for functions *in vivo*.

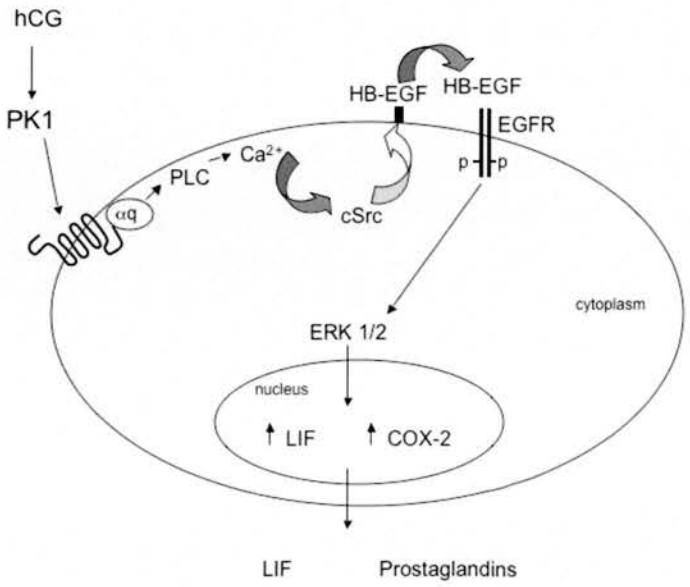


Figure 9.1. Schematic representation of PROK1 mediated signalling and gene activation. PROK1 via PROKR1 activates its Gq coupled receptor and activates intracellular signalling molecules inducing phosphorylation of cSrc, EGFR and ERK 1/2. Downstream of ERK 1/2 phosphorylation, PROK1 mediates expression of a number of genes, including LIF and COX-2. PROK1 subsequently induces production of LIF protein and prostaglandins. In the event of pregnancy, PROK1 expression is positively regulatd by embryonic hCG.

Elevation of PROK1 during the secretory phase of the cycle is regulated by progesterone (Battersby *et al*, 2004a, Ngan *et al*, 2006). Progesterone secretion by the corpus luteum is maintained in early pregnancy by luteotrophic hCG. PROK1 is significantly elevated in early pregnancy decidua compared with non-pregnant endometrium. It is suggested herein that this is in response to embryonic hCG, rather than solely a continued response to progesterone (Figure 9.1). It is now appreciated that hCG has a direct effect on the endometrium (Licht *et al*, 2001, Perrier d'Hauterive *et al*, 2004, Sherwin *et al*, 2007), and has been demonstrated to regulate expression of endometrial LIF (Licht *et al*, 2001, Sherwin *et al*, 2007). The data presented in this thesis demonstrate that PROK1 is regulated by hCG in PROKR1 Ishikawa cells, first trimester decidua and an *in vivo* baboon model. Furthermore, hCG induced LIF expression appears to be dependent on expression of PROK1, illustrated

by the temporal delay between PROK1 expression and LIF expression, and the inhibition of hCG induced LIF expression by an inhibitor of protein synthesis.

9.1.4. Relevance to implantation and early pregnancy

9.1.4.1.Human endometrial gene expression

The regulation of PROK1 during the secretory phase of the menstrual cycle and regulation by progesterone points towards a potential role for PROK1 in endometrial receptivity. PROK1 is specifically elevated during the mid-secretory phase of the menstrual cycle, a period known as the window of implantation (Battersby *et al*, 2004a, Ngan *et al*, 2006). The results of this thesis have made further advances and demonstrate that PROK1 regulates genes suggested to play roles in endometrial receptivity and are expressed in the human endometrium during this time. These genes include CD44, DAF, Dkk-1, HB-EGF, GADD45A, IL-11, IL-6, LIF, Metallothioneins, COX-2, and the TGFβ superfamily. DAF, Dkk-1, GADD45A, the metallothioneins and TGFβ superfamily members have been shown, by gene array analysis, to be elevated in the receptive endometrium (Kao *et al*, 2002, Carson *et al*, 2002, Riesewjik *et al*, 2003, Borthwick *et al*, 2003, Mirkin *et al*, 2005, Talbi *et al*, 2006). Other proteins, such as HB-EGF, CD44, IL-6 and also LIF and IL-11 have been implicated in endometrial receptivity due to their expression across the menstrual cycle in humans, with an elevation in the secretory phase of the cycle (Leach *et al*, 1999, Yoo *et al*, 1997, Stavreus-Evers *et al*, 2002, Horne *et al*, 2002, Afify *et al*, 2006, Tabibzadeh *et al*, 1995, Vandermolen and Gu, 1996, Dimitriadis *et al*, 2002, 2003, Arici *et al*, 1995, Cullinan *et al*, 1996, Charnock-Jones *et al*, 1994, Vogiagis *et al*, 1996).

9.1.4.2. Gene knockout studies

A number of PROK1 regulated genes have proposed roles in reproductive function due to the phenotypes of mice null for these genes. LIF null mice do not implant or display a decidualization reaction (Stewart *et al*, 1992, Chen JR *et al*, 2000) due to a maternal defect in LIF production. COX-2 null mice display a defect in decidualization and a delay in parturition due

to an absence in endometrial prostaglandin production (Cheng & Stewart, 2003, Lim *et al*, 1997, Matsumoto *et al*, 2002). IL-11 Rα null display defective decidualization and death of embryos in utero (Robb *et al*, 1998, Bilinski, 1998), while IL-6 null mice are reported to exhibit a 48% reduction in implantation (Robertson *et al*, 2000). Interestingly, a number of implantation related genes, including HB-EGF, Amphiregulin, COX-2 and HOXA-10 are aberrantly expressed in the LIF null mouse (Song *et al*, 2000, Nashta-Fouladi *et al*, 2005), potentially suggesting LIF to be at the top of a signalling cascade mediating implantation.

9.1.4.3.Endometrial pathologies

Examination of gene expression in endometrial pathologies associated with implantation defects or inadequate endometrial receptivity have provided further information pertaining to genes required for successful pregnancy.

DAF, or CD55, is elevated in the receptive endometrium, as demonstrated by gene array analysis (Kao *et al*, 2002, Carson *et al*, 2002, Riesewijk *et al*, 2003, Borthwick *et al*, 2003, Mirkin *et al*, 2005). DAF is down-regulated in women with antiphospholipid syndrome, a condition associated with a high miscarriage rate (Francis *et al*, 2006).

Aberrant expression of PROK1 regulated genes in pathological disorders, such as a reduction in DAF expression in women who suffer from antiphospholipid syndrome (Francis *et al*, 2006), may indicate a requirement for examination of PROK1 expression in this disorder. LIF production is suggested to be reduced in women who suffer from unexplained infertility (Laird, 1997 *et al*, Hambartsoumian, 1998b). IL-6 expression is also suggested to be reduced in women who suffer from recurrent miscarriage (Jasper *et al*, 2007). Dkk-1 is proposed to be down-regulated in endometriosis (Kao *et al*, 2003) As LIF, IL-6 and Dkk-1 are PROK1 regulated genes, examination of PROK1 expression in women with unexplained infertility, recurrent miscarriage or endometriosis may reveal a reduction in the level of PROK1.

9.1.4.4.Trophoblast function

Examination of the genes regulated by PROK1 reveals elevation of a group of genes which are proposed to be pro-invasive and a group of genes, which are proposed to be anti-invasive. The overall outcome of these genes would presumably depend on the surrounding endometrial milieu governing activation or inactivation of these gene products.

PROK1 elevates expression of adrenomedullin. Adrenomedullin has been suggested to enhance invasion of the trophoblast, specifically maternal expression of adrenomedullin is suggested to play a role in implantation and placentation (Zhang X *et al*, 2005, Li M *et al*, 2006). The elevation of COX-2 expression by PROK1 has been demonstrated herein to induce down-stream production of prostaglandins. PGE_2 is proposed to enhance trophoblast invasion through activation of calpain 2, another PROK1 regulated gene (Nicola *et al*, 2005). Calpains are protease enzymes, which aid cell migration (Dourdin *et al*, 2001, Huttenlocher *et al*, 1997, Glading *et al*, 2000). PROK1 also regulates expression of other proteases including MMP-10 and uPA. PROK1 also elevates expression of the receptor for uPA, which is essential for its activity. However, PROK1 elevates PAI-1, the inhibitor of uPA, thereby potentially providing a control mechanism.

In contrast to the pro-invasive genes regulated by PROK1, a number of anti-invasive genes are also elevated. Activin A is suggested to stimulate trophoblast migration (Caniggia *et al*, 1997), however, PROK1 elevates expression of its inhibitor, Follistatin. This may imply PROK1 has an inhibitory effect on trophoblast invasion. PROK1 also elevates expression of TGF-β3, which inhibits trophoblast outgrowth and invasion (Caniggia & Winter, 2002, Caniggia *et al*, 2000). It is also elevated in syndromes of deficient placental invasion such as pre-eclampsia (Caniggia *et al*, 1999), potentially suggesting PROK1 to be involved in the pathogenesis of pre-eclampsia. A recent study has suggested that no differences exist in the expression of PROK1 between pre-eclamptic and normal placentas (Chung *et al*, 2004). This study was conducted on placentas collected at term. However, as the pathogenesis of pre-eclampsia starts in the first trimester

of pregnancy, potential effects of PROK1 in the initiation of this disease may be masked in advanced gestation.

9.1.4.5.Embryonic signals

Regulation of PROK1 by hCG, and the expression of PROK1 and PROKR1 in first trimester decidua implies a continued role for PROK1 during early pregnancy, after the initial implantation reaction. hCG has been demonstrated to elevate expression of a number of PROK1 regulated genes, including COX-2, LIF and IL-6 (Perrier d'Hauterive *et al*, 2004, Sherwin, 2007 *et al*, Licht *et al*, 2001, Han *et al*, 1996, Zhou *et al*, 1999). This thesis suggests that hCG may regulate expression of these genes via induction of PROK1. LIF and IL-6 have a reciprocal effect on the trophoblast, stimulating hCG production, and it may be assumed syncytialisation, as hCG production takes place as a function of syncytialisation (Matsuzaki *et al*, 1995, Sawai *et al*, 1995a, 1995b, Yang *et al*, 2003).

Whether PROK1 enters into a similar reciprocal relationship with hCG is not yet known. However, as demonstrated in Chapter 3, the trophoblast does express PROKR1. It is suggested that early placental expression of PROKR1 peaks in weeks 8 - 10 of pregnancy (Hoffmann *et al*, 2006). This may therefore provide a mechanism whereby endometrial PROK1 may communicate with the invading trophoblast.

9.1.5. Potential therapeutic uses for prokineticins or prokineticin inhibitors

It has been demonstrated that simple light microscopy examination of the endometrial histology provides little information regarding the receptivity of the endometrium (Saleh *et al*, 1995). Therefore molecular markers would aid the definition. No single marker of uterine receptivity has thus far been identified. A number of candidates, such as LIF, IL-11, HB-EGF, $\alpha v\beta 3$ integrin and IL-6 have been suggested as markers of uterine receptivity. However, it is likely that no one factor will define the period of receptivity in all women. Examination of a number of markers used in concert should help to better identify the receptive endometrium. In this study, PROK1 is proposed as a

potential marker of uterine receptivity. PROK1 is elevated in the endometrium during the secretory phase of the cycle, which encompasses the window of implantation, and is further elevated during pregnancy. PROK1 also regulates a number of implantation related genes, suggesting it may act as a 'master controller' in the endometrium during the receptive phase. Expression is also regulated by hCG, a hormone produced by the blastocyst and syncytiotrophoblast during early pregnancy and routinely given during IVF cycles (Hoult *et al*, 1981, Jones *et al*, 1982). hCG induced PROK1 may therefore contribute to endometrial receptivity during stimulated cycles.

As PROK1 appears to regulate expression of endometrial receptivity and implantation related genes it is plausible that delivery of PROK1 during stimulated cycle for IVF may aid receptivity. However, the route of delivery would have to be investigated, as the systemic effects of PROK1 are not yet known. PROK1 has been demonstrated to induce gastrointestinal (GI) contractions or relaxation, depending on which section of the GI tract was examined (Li M *et al*, 2001, Hoogerwerf, 2006). This may be a complication if systemic delivery of PROK1 were used. If it were possible to administer PROK1 vaginally, a more localised effect of PROK1 may be observed. Currently, the physiological concentration of PROK1 is unknown. Furthermore, no ELISA is available at present to measure the concentration of PROK1 in fluids such as endometrial lavage fluid. Therefore, the concentration of PROK1 that may be used in such treatments is currently unknown. This is an important consideration as PROK1 may induce uterine contractions. PROK1 may induce contractions directly by acting on the uterine smooth muscle, which express PROKR1 (Battersby *et al*, 2004a), as observed in the gastrointestinal tract (Li M *et al*, 2001). PROK1 may also induce contractions indirectly by elevating genes such as COX-2 and downstream production of prostaglandins. Prostaglandins have been demonstrated to stimulate uterine contractions (Friel *et al*, 2005) and synthetic prostaglandins are used to induce early first trimester abortions (Bjorge *et al*, 2001, Bygdeman *et al*, 2000, Bygdeman *et al*, 1994). Therefore, the incorrect concentration of PROK1 may be detrimental to the successful establishment and maintenance of pregnancy.

If PROK1 mediates endometrial receptivity to an implanting embryo, then inhibitors of PROK1 action, such as receptor antagonists, may cause the endometrium to become refractory to implantation. This may provide a novel contraceptive with fewer side effects than traditional hormonal contraceptives.

9.2. Conclusions

In conclusion, this thesis has demonstrated the temporal expression across the menstrual cycle of PROK1 and PROKR1. Expression and localisation of both factors has also been examined in the pregnant and pathological endometrium. PROK1 displays elevation during the secretory phase of the cycle, with both PROK1 and PROKR1 elevated in the pregnant endometrium. However, no abberation in expression of these factors is observed in endometrial adenocarcinoma. Signalling via PROK1 induces activation and phosphorylation of multiple signalling molecules including cSrc, EGFR and ERK 1/2. Furthermore activation of PROKR1 mediates expression of a number of genes, two of which, COX-2 and LIF, were investigated further. Expression of both COX-2 and LIF is induced by PROK1 in a temporal manner with expression dependent on activation of Gq - PLC-β - Ca^{2+} - cSrc - EGFR and MEK. PROK1 expression is induced by hCG, and hCG mediated LIF expression may be dependent on induction of PROK1. These data outline a possible function for PROK1-PROKR1 interaction in contributing to endometrial receptivity and implantation through induction of factors proposed to be necessary for the initiation of pregnancy. PROK1-PROKR1 in the first trimester of pregnancy, may contribute to the continued growth and development of the placenta.

9.3. Future research direction

The data presented herein have characterised the signalling and gene activation induced by PROK1 via PROKR1. The genes regulated by PROK1 include a number of genes proposed to play a role in implantation. Thus, it is proposed that PROK1-PROKR1 plays a role in endometrial receptivity and implantation.

The role of PROK1-PROKR1 in endometrial receptivity and implantation has, therefore, to be defined. Does PROK1 signalling via PROKR1 act as a master controller of implantation downstream of hormonal regulation? A PROKR1 knockout mouse has been produced by Matsumoto *et al* (2006), however, the reproductive phenotype for this mouse is not yet known. In the absence of a reproductive phenotype it may be proposed that PROK1 signalling through PROKR2 may compensate for the absence of PROKR1. The PROKR2 knockout mouse does not have a functional reproductive system due to the non-development of GnRH neurones and therefore gonadotropin support for the reproductive tract. Use of a knockout mouse model for PROK1 or specific inhibitors of PROK1-PROKR1 induced signalling may help answer the question of whether PROK1 plays a role in implantation. In order to decipher the role of the two prokineticin receptors in implantation, a PROKR1 null/PROKR2 conditional endometrial knockout mouse model or PROKR1 null/PROKR2 null with exogenous gonadotropin support may be used. These models should help delineate the presence or absence of compensatory signalling (ie. PROK1 signalling via PROKR1 and/or PROKR2) and determine the role of prokineticin signalling in implantation.

Knockout rodent models provide useful tools with which to study the physiological role of genes *in vivo*. However, caution should be applied when extrapolating data from mouse models into human implantation. A number of genes, such as LIF, expressed during the secretory phase of the cycle in the human endometrium are only expressed after the nidatory estrogen surge on day 4 of pregnancy in mice (Bhatt *et al*, 1991). This dependence on estrogen represents a difference between humans and rodents, as humans are not dependent on this surge of estrogen for implantation. Decidualisation in the mouse endometrium demonstrates differential regulation compared with the human endometrium.

Decidualization in the mouse is mainly dependent on PGI_2, with signalling occurring via PPARδ (Lim *et al*, 1999, Lim *et al*, 1997, Barak *et al*, 2002). However, decidualization in the human is suggested to occur via PGE_2 induction of Camp (Dimitriadis *et al*, 2005, Frank *et al*, 1994), and the human endometrium produces little PGI_2 (Abel & Kelly, 1979). Although both humans and mice exhibit haemochorial placentation, differences exist in the depth of trophoblast invasion between the two species, with human trophoblasts being highly invasive. Mouse models provide a useful *in vivo* system in which implantation and associated decidualization and placentation can be studied in more detail with experimental manipulation. Other model systems in which implantation, decidualization and placentation may be studied, with closer parallels to the human, include non-human primates such as the baboon. Data presented in this thesis demonstrate that treatment of baboon endometrium with hCG induces expression of PROK1, potentially indicating a role for endometrial PROK1 in pregnancy. Hence, application of antagonists directed against PROKR1, or inhibitors of PROK1-PROKR1 mediated signalling, may be useful in understanding the role of PROK1 in primate implantation and pregnancy.

It is suggested herein that hCG mediated LIF expression is dependent on expression of PROK1. However, this has only been investigated using a general inhibitor of protein synthesis. These studies should therefore be extended using an inhibitor of PROK1 or an siRNA against PROK1 in order to further determine its role in regulating hCG mediated LIF expression.

Factors induced by PROK1, including HB-EGF, LIF, COX-2, CD44 and galectins are proposed to play roles in mediating blastocyst attachment to the luminal epithelium. It may be proposed therefore, that PROK1 may play a role in blastocyst attachment via induction of these proteins at the endometrial – blastocyst interface. Indeed, preliminary studies, using adhesion assays and choriocarcinoma cells, have suggested a role for PROK1 induced products in mediating adhesion of trophoblast cells to extracellular matricies. Hence the mechanism by which prokineticin regulates cellular adhesion warrants further research.

The potential role of PROK1 and PROKR1 in infertility should be examined. As discussed in Section 9.1.4.3, expression of PROK1 regulated genes, including DAF, LIF and IL-6, are down regulated in endometrial pathologies associated with infertility. DAF expression is down-regulated in antiphospholipid syndrome, a condition associated with a high miscarriage rate (Francis *et al*, 2006). LIF and IL-6 are down-regulated in women with unexplained infertility and recurrent miscarriage (Laird *et al*, 1997, Hambartsoumian, 1998b, Jasper *et al*, 2007). Endometrial PROK1 and PROKR1 expression should therefore be examined in these conditions in order to determine the expression pattern in infertile women or those suffering from recurrent miscarriage. If PROK1 expression or signalling is dysfunctional in unexplained infertility or spontaneous abortion, treatment regimes may be developed in order to deal with these targets. Such treatment may improve the probability of successful pregnancy.

Assuming PROK1 does play a role in implantation and early pregnancy events, the next question to be answered is what physiological processes it regulates at this time. PROK1 induces expression of LIF. LIF has been demonstrated, via induction of hCG to contribute to the formation of the placental syncytium (Yang *et al*, 2003, Sawai *et al*, 1995b). As hCG induces expression of PROK1 this may indicate a positive feedback loop for the induction of syncytialisation. PROK1 also induces expression of COX-2. hCG mediates COX-2 expression during the differentiation of human endometrial stromal cells into decidua (Han *et al*, 1996). Thus PROK1 may play a role in syncytialisation of the trophoblast and decidualization of the endometrium via induction of these genes. This may suggest a role for PROK1 in limiting trophoblast invasion, as syncytiotrophoblasts are not invasive and decidua produces inhibitors to the proteases produced by the trophoblast (Alexander *et al*, 1996, Afonso *et al*, 1997). Indeed, in the absence of appropriate decidua formation the trophoblast proliferation is unrestrained (Robb *et al*, 1998).

PROK1 elevates expression of a number of pro-invasive and anti-invasive genes. It would therefore be of interest to investigate which of these groups would display dominance and whether the net effect would be invasion or inhibition of invasion. However, any studies *in vitro* examine only one factor in isolation, whereas *in vivo*,

the surrounding milieu would enhance or inhibit factors. The net effect *in vivo* may therefore be different to that observed *in vitro*. Preliminary studies have been performed by Alfaidy *et al* (89th Endocrine Society meeting, 2007). This group reports that PROK1 inhibits the migration and invasion of extravillous trophoblasts while increasing the proliferation of the cytotrophoblasts, suggesting that PROK1 may regulate placental development in the first trimester. If PROK1 does indeed have an anti-invasive effect it may be postulated that this protein has a role in conditions associated with the under-invasion of the trophoblast such as the pathogenesis of pre-eclampsia and intrauterine growth restriction. A study examining the PROK1 in term placenta from pre-eclamptic pregnancies found no differences in expression of PROK1 between these and normal pregnancies (Chung *et al*, 2004). However, as the pathogenesis of these conditions may arise from defective invasion during the first trimester, differences in PROK1 expression in early pregnancy may be more indicative of the initiation of the disease as discussed in Section 9.1.4.4. The onset of pre-eclampsia is not detected until week 20 of pregnancy, during the second trimester. Therefore, pre-eclamptic first trimester trophoblast samples are unavailable as the pregnancy is terminated before week 12 of gestation, and diagnosis of pre-eclampsia is not made until week 20. The potential role of PROK1 in pre-eclampsia may therefore be investigated using animal models of induced pre-eclmapsia (Karumanchi *et al*, 2006). The administration of PROK1 during various stages of pregnancy in the mouse may also shed some light on the effect of PROK1 on placental development during pregnancy.

PROK1 is expressed in term placenta (LeCouter *et al*, 2001, Hoffmann *et al*, 2006, Chung *et al*, 2004, Li M *et al*, 2001). In light of the reported effects of PROK1 in inducing contractions in smooth muscle it would be interesting to investigate the expression of PROK1 and PROKR1 in term chorionic membranes. As this study has demonstrated, PROK1 can induce expression of COX-2 and production of downstream prostaglandins. Prostaglandins have a proven role in parturition, during which time COX-2 expression is elevated (Friel *et al*, 2005, Slater *et al*, 1994). Other factors induced by PROK1, including IL-8 are suggested to play a role in parturition (Maul *et al*, 2002). This may suggest a role for PROK1 signalling

throughout human pregnancy, during the initiation of pregnancy at the time of implantation and at the termination of pregnancy at parturition.

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